

DEVELOPMENT OF A RAPID RIBOFLAVIN GROWTH-BASED ASSAY
USING *Lactobacillus rhamnosus*

A Thesis

by

JENNIFER L. GOLBACH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Poultry Science

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ABSTRACT

Development of a Rapid Riboflavin Growth-Based Assay Using *Lactobacillus*
rhamnosus.

(December 2005)

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Riboflavin is an essential part of the human diet. Although the United States does not have a major problem with a riboflavin deficiency, other regions of the world do. This is especially true for those regions whose main subsistence is rice. To help prevent and control riboflavin deficiencies, many cereal grains are now being fortified with riboflavin. The recommended dietary allowance of riboflavin is 1.1-1.6 mg per day. This value increases slightly for pregnant women, breast feeding women, and athletes. Because riboflavin is an essential part of the diet, it is important to ensure that the minimum requirements for this nutrient are met. By determining the amount of riboflavin in food products, an accurate estimate of daily riboflavin intake can be determined. The AOAC (Association of Official Analytical Chemists) approved microbiological riboflavin assay can be tedious and time consuming. A faster approach to the riboflavin assay would greatly benefit the food industry. By scaling down the assay to microtiter plates both, time and materials can be conserved. Use of microtiter plates would also allow for numerous samples to be assayed simultaneously. The goal for developing the microtiter plate assay is to obtain results more rapidly while maintaining the accuracy and precision of the AOAC (method 940.33I) tube assay.

DEDICATION

I would like to dedicate my thesis to my parents. They have always been there for me, and have always encouraged me to do my best at anything I tried. It was hard moving so far away, but I always knew I could give them a call anytime I needed anything.

To my sister, Lindsay, with whom I have not always gotten along with, but have come to share a special bond with now that we are both able to overlook the small things.

To Rhianna who has been there for me through thick and thin. Whenever I am having a hard time, she is always there to put a smile on my face.

I would also like to thank the rest of my family, friends, and neighbors who have always believed in me and encouraged me throughout my life. It really helped knowing there are so many people in my life that are there to give me encouragement and inspiration.

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CHAPTER I

INTRODUCTION

Riboflavin, also known as vitamin B₂, is an important dietary requirement for the humans and animals. It is essential for normal growth and development as well as the production and regulation of certain hormones and the formation of red blood cells. It is also important for the cellular metabolism and the production of biological energy through the electron-transport system (Choe et al., 2005). Although plants are capable of synthesizing riboflavin, animals are not capable of synthesizing or storing large amounts riboflavin, so there is a constant need for it to be included in the diet (Cataldi et al., 2002).

The most practical approach for quantifying the amount of riboflavin in samples is the microbiological assay utilizing *Lactobacillus rhamnosus*. This procedure has the advantage of reducing space, time, equipment, and trained personnel over other analytical techniques such as HPLC (high performance liquid chromatography), fluorescence, and animal studies (Voigt and Eitenmiller, 1985). Also, *L. rhamnosus* is non-pathogenic thus there are little concerns for working with it in the laboratory (Sneath et al., 1986). By making adaptations of the microbiological tube assay to microtiter plates, more samples can be assayed at one time. The microtiter plate adaptation will also save on materials and labor.

Studies to determine the amount of riboflavin in food sources as well as animal feeds are important to ensure that the appropriate quantity is available for consumption.

This thesis follows the style of the Journal of Food Science.

Although deficiencies in the United States are rare, inadequate intake in other geographical areas is a problem where the main staple is rice. Fortification of cereals can greatly reduce riboflavin deficiency in these areas (McNulty et al., 1996).

CHAPTER II

LITERATURE REVIEW

History of Riboflavin

In the early part of the twentieth century, “factor B” was found in the watery part of milk that was essential for animals fed a purified diet that consisted of purified fat, carbohydrate, protein, and mineral salts. “Factor B” was found to be an amine from which the name “vitamine” was coined and which stood for “vital amines” (Bender, 2002). Further studies showed that not all vitamins were chemically made up of amines so the “e” on the end of the word was dropped (Bender, 2002). The factor B was discovered to be a mixture of a number of compounds that performed different functions in the body. One of these compounds was riboflavin or vitamin B₂ (Bender, 2002).

When riboflavin was first discovered, it was named according to the food component it was extracted from. When discovered in milk, it was referred to as lactoflavin, and when discovered in eggs, it was referred to as ovoflavin. Later, it was called vitamin G because of the green fluorescent pigment isolated from whey, and finally, it was called vitamin B₂ or riboflavin (Northrop-Clewes and Thurnham, 2002 ; Carpenter, 2003). The accepted chemical name for riboflavin is 7,8-dimethyl-10-1'-D-ribityl-isoalloxazine. The flavin ring is methylated at positions 7 and 8 and contains a D-ribityl moiety at position 10 (Eitenmiller and Landon, 2000). Riboflavin was discovered as a “yellow growth factor” in the heat-stable portion of antipellegra extracts. In 1935, it was synthesized in the lab, and by 1938, its structure was determined (Villamor et al., 2003). Commercial riboflavin is either generated by chemical synthesis or microbial

fermentation, the latter of which is the most common and is used to produce most of the riboflavin (Choe et al., 2005).

History has shown that diet has been a common way to prevent and treat ailments such as rickets, night blindness, pellagra, and scurvy, but it was not until the end of the nineteenth century that specific compounds in foods were determined to be vital for normal bodily functions. Vitamins have traditionally been identified as being either fat soluble such as vitamin A or water soluble such as riboflavin (Villamor et al., 2003).

Compounds are confirmed to be vitamins by proving that a deficiency will cause a specific deficiency disease and/or abnormal metabolic signs. Also, proof is needed that demonstrate that when the vitamin is restored to the diet that the symptoms of the disease and/or metabolic abnormalities disappear (Bender, 2002). It is not enough proof that a compound is a vitamin if it performs a specific metabolic function in the body or that it cures a disease because this could simply mean that the compound has a pharmacological action and does not mean that it is necessarily essential in the diet (Bender, 2002).

Effects of Food Processing

Processing and storage of food can significantly affect the quantities of vitamins found in dietary sources. The factors that can affect vitamins vary depending on the vitamin. The principal factors that affect vitamins in food are heat, moisture, oxygen, pH, and light (Ottaway, 2002). One or more of these factors can be encountered using various processing methods such as milling, fermentation, germination, extrusion, and thermal processing (Reddy and Love, 1999). These factors affect vitamins in the same manner regardless of whether the vitamins occur naturally or if they are added.

However, the form in which a synthetic vitamin is added may enhance the stability of the vitamin (Ottaway, 2002).

Riboflavin is regarded as one of the more stable vitamins because both riboflavin and riboflavin-phosphate are both stable to heat and atmospheric oxygen, especially in acidic media. However, riboflavin is especially sensitive to sunlight and to a slightly lesser extent to fluorescent light particularly in a liquid medium. It is also increasingly unstable as the pH rises (Ottaway, 2002).

Many of the foods that are consumed by humans are partially or fully processed before they are displayed at retail and may undergo additional processing in the home prior to consumption. Although processing of food can make it healthier, safer, tastier, and more shelf-stable, it can also reduce vitamins and other nutrients (Reddy and Love, 1999). Riboflavin is a relatively stable vitamin during dehydration, γ -radiation, and under various storage conditions, but it is affected by oxygen, metal sulfates, amino acid chelates, and water activity. Riboflavin is normally protected from γ -radiation by prosthetic groups when riboflavin is bound to proteins. The destruction of riboflavin increases dramatically when oxygen is present during storage as well as when metal sulfates are present. Water activity also has an effect on riboflavin. Almost 100% of riboflavin is retained with moisture storage, but the loss of riboflavin increases as water activity increases (Choe et al., 2005).

A study done by Prodanov et al. (2004) determined riboflavin loss in legumes when soaked and cooked. The results showed that soaking in sodium bicarbonate significantly decreased the riboflavin content of faba beans and chickpeas. This also was the case for those soaked in sodium bicarbonate and, then, cooked. Lentils, on the other hand, had a marked increase of riboflavin content when soaked in citric acid, water, or

sodium bicarbonate. The only decrease of the riboflavin content was when the lentils were soaked in sodium bicarbonate and, then, cooked. The increase in lentil riboflavin content can be explained by the microbial activity found in the lentil cover. Immediately after humidification of lentil seeds, bacteria, mainly certain *Lactobacillus* species, are able to synthesize riboflavin (Prodanov et al., 2004).

Thermal processing of soymilk was studied to determine whether there was an effect on riboflavin content. Many Asian countries drink more soymilk than dairy milk, and each can provide a significant amount of dietary riboflavin. The amounts of riboflavin in soymilk are comparable to those in dairy milk. A study done by Kwok et al. (1997) revealed that thermal processing has an insignificant effect on the riboflavin content of soymilk.

Food Fortification

Fortification of foods with riboflavin is sometimes necessary when the foods commonly eaten in different parts of the world do not provide enough of the vitamin to prevent a deficiency. The most common foods fortified with riboflavin are breakfast cereals. Studies show that riboflavin intake increases with increased consumption of breakfast cereals for all age groups. This indicates that the fortification of breakfast cereals is beneficial, especially to populations that do not consume a balanced diet rich in certain vitamins such as riboflavin (McNulty et al., 1996). There is strong evidence that significant population subgroups have suboptimal intakes of certain vitamins and minerals such as iron, calcium, zinc, vitamins B1, B2, B6, D, and folate. Studies have shown that mandatory and voluntary addition of nutrients to foods can help ease the nutrient deficiency problem in at-risk populations (Flynn et al., 2003). Up to 200

mg/serving of riboflavin, the determined maximum safe addition level for this particular vitamin, can be safely added to foods (Flynn et al., 2003). Riboflavin is considered a category C vitamin because there have been no adverse affects observed with excess intake. The safe upper intake range is almost impossible to determine due to the absence of toxicity even at 100 times the RDA (recommended dietary allowance). Other vitamins that share this category are vitamin K, thiamine, pantothenic acid, and biotin (Meltzer et al., 2003).

Because there is a loss of vitamin content during processing and consumption of ready-to-eat foods has increased, there is an increasing need to fortify more foods with essential vitamins (Agte et al., 2002). Although fortification is not a replacement for a balanced diet, it does help alleviate some of the nutritional deficiencies that some parts of the world's population have.

A recent study has shown that B-group vitamin supplementation, which includes riboflavin, to stroke victims has antioxidant and anti-inflammatory effects as well as a homocysteine-lowering effect. These are all positive effects jointly associated with riboflavin that benefit stroke victims. By supplementing riboflavin along with the other B-group vitamins during a critical 12 hour period after a stroke enhances antioxidants which mitigates oxidative damage to stroke patients (Ullegaddi et al., 2004).

Forms of Riboflavin

Riboflavin is a building block for certain essential flavin coenzymes such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)(figure 2.1). These flavin coenzymes act as prosthetic groups for flavoprotein enzymes (Hoppel and Tandler, 1990). Although riboflavin may be ingested as riboflavin, once it enters the body, it can

be converted to other biological forms such as FMN or FAD. This occurs when riboflavin is phosphorylated (Fogelholm, 2000). The phosphorylation of riboflavin to FMN in mammalian tissues is catalyzed by a Zn^{2+} preferring flavokinase (McCormick, 2003a). The structures of riboflavin, FMN, and FAD are composed of an isoalloxazine ring with a ribityl side chain at position 10. The modifications in the ribityl side chain are what distinguishes riboflavin from FMN and FAD (Cataldi et al., 2002 and Fogelholm, 2000). The ribityl side chain is required for full vitamin activity, and it is the alcohol form of the 5-carbon sugar, ribose (Trusswell and Milne, 1998).

FMN and FAD function in numerous glycolysis and respiratory chain oxidation-reduction reactions. They are required for reaction catalyzations, dehydrogenation, and oxidative decarboxylations as well as being involved in the respiratory chain, lipid metabolism, the cytochrome P-450 system, and drug metabolism (Rivlin, 2002). FMN is needed for the synthesis of fatty acids from acetate whereas Figure 2.1: Different forms of riboflavin present in food sources and tissues. FAD is required for fatty acid oxidation (Fogelholm, 2000). Rats fed riboflavin-deficient diets showed abnormal lipid metabolism as well as a reduction in the beta-oxidation of fatty acids (Witte and Clark, 2004). FAD is also required for the pyruvate dehydrogenase complex, α -ketoglutarate dehydrogenase complex, and succinate dehydrogenase which are enzymes involved in the citric acid cycle (Fogelholm, 2000). FAD is the most common biological form of riboflavin in tissues and circulation (Powers, 1999).

Nutrition

Riboflavin is found in a wide range of dietary sources. The best sources are milk and dairy products, eggs, liver, kidney, yeast extracts, and fortified cereals (Trusswell and

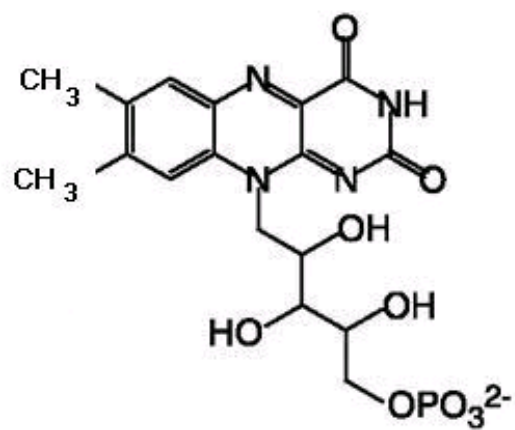
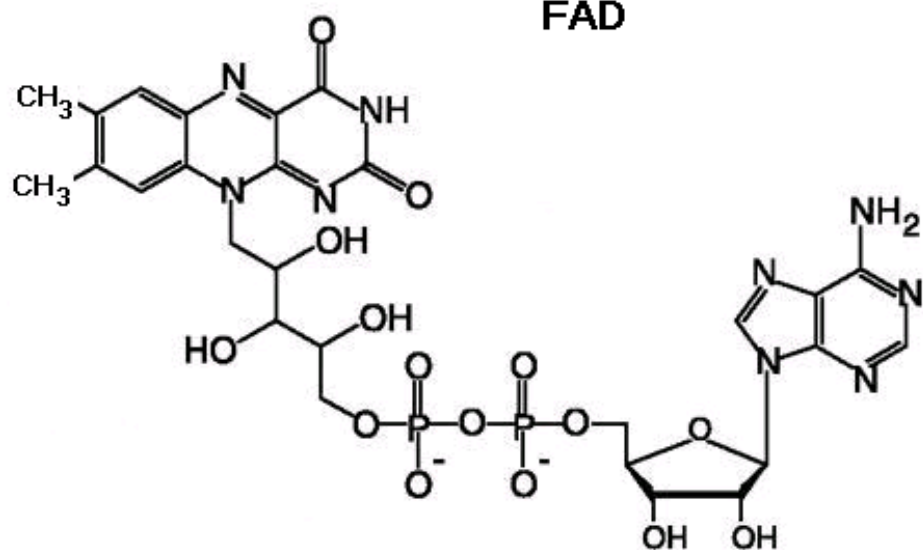
Riboflavin**FMN****FAD**

Figure 2.1: Different forms of riboflavin.

Milne, 1998). Even though a significant contribution of riboflavin is provided through milk consumption, riboflavin is light sensitive and milk loses 70% of its riboflavin content when exposed to at least 4 hours of ultraviolet light (Truswell and Milne, 1998). Milk stored in opaque or other containers that do not let light through do not lose as much of the vitamin. Animal sources have been found to provide a better source of riboflavin than natural plant sources (Murphy, 2003; Murphy and Allen, 2003).

The dietary requirements for most vitamins, including riboflavin, have been calculated and are referred to as the recommended dietary allowance (RDA) (Northrop-Clewes and Thurnham, 2004). Because dietary requirements vary between individuals, this number represents an amount of riboflavin that should be sufficient to meet the metabolic needs of at least 95% of the population (Northrop-Clewes and Thurnham, 2004). Riboflavin requirement estimates are based on depletion/repletion studies. These studies are used to determine the minimum amount of riboflavin intake at which there is no significant excretion of the vitamin in the urine. The minimum adult requirement of riboflavin is 0.5–0.8 mg/day where there is very little excretion in the urine. When the intake of riboflavin reaches 1.1–1.6 mg/day, the urinary excretion rises sharply indicating that the tissue reserves are saturated (Bender, 2002). An additional 0.3 mg/day is recommended during pregnancy while an additional 0.5 mg/day is recommended during lactation (Manore, 2000). The main advantage to the urinary excretion assay is the existence of a “breakpoint” which can be used to determine the tissue saturation point and define the body’s requirements with a fair amount of accuracy. This approach has never been very popular in practice because it has been difficult to get sufficient subject cooperation with the timed collection of urine samples as well as being a poor

representation of riboflavin status between the ranges of mild to severe desaturation (Bates, 1993).

Another way to determine riboflavin status in the body is the red blood cell riboflavin pool which gives an estimate of intracellular riboflavin levels. Most of the riboflavin is located in the cells, especially the erythrocytes, which have four to five times more total flavin than the serum (McCormick, 1989). Also, the flavin levels in erythrocytes are not influenced as much by the recent intake of riboflavin, so tests utilizing erythrocytes give a more accurate riboflavin reading (McCormick, 1989). This assay has not achieved the popularity of the glutathione reductase index mainly because tissue saturation is more difficult to define and the saturation values have a tendency to vary between individuals (Bates, 1993).

Riboflavin status in the body can also be determined by using the activity and activation coefficient of glutathione reductase which is the red cell FAD-dependent enzyme. Erythrocyte glutathione reductase (EGR) requires not only NADPH, but also, FAD. This enzyme regenerates reduced glutathione from its oxidized form. EGR performs a critical role in finding potentially harmful intracellular reducing equivalents. Under normal circumstances, EGR is not saturated with FAD coenzyme which has led to the development of biochemical assays to determine the extent of erythrocyte riboflavin depletion (Ross and Hansen, 1992). The “enzyme functional index” of riboflavin which is often referred to as EGRAC (Erythrocyte Glutathione Reductase Activation Coefficient) assay is determined by the ratio of enzyme activities with and without the added cofactor of FAD. This approach is closely linked to the red cell riboflavin pool assay (Bates, 1993). This is the most widely used assay to determine riboflavin tissue saturation because the biological materials are relatively stable when stored frozen, only

small amounts of blood are needed, it is discriminatory within the normal range of values, and it provides an accurate reflection of tissue saturation over a wide range of status values. The one disadvantage of this test is that it fails to quantitate riboflavin concentrations when glucose 6-phosphate dehydrogenase deficiency is also present (Bates, 1993).

Chemistry

Oxidation/reduction reactions

Riboflavin is a major part of FMN and FAD, which catalyze a wide range of oxidation-reduction reactions. Riboflavin is able to accept or donate a pair of hydrogen atoms which makes it an easy candidate for oxidation or reduction. Flavins are capable of one or two electron transfers which play a major role in the coupling of the two-electron oxidation of most organic substrates to the one-electron transfers of the respiratory chain. The one-electron transfers to various metal substrates are conducted through their free radical state (Massey, 2000). Flavins are able to form portions of redox-center enzymes through their free radical states. Some examples are succinate and NADH dehydrogenases, xanthine oxidase/dehydrogenase, cytochrome P450 systems, and nitric oxide synthase (Massey, 2000). This ability to donate and accept electrons also makes riboflavin a highly susceptible target for photochemistry alterations by exposure to light. When irradiated under visible or UV light, riboflavin can produce superoxide radicals, singlet oxygen, hydroxy radicals, and hydrogen peroxide when atmospheric oxygen is present (Choe et al., 2005). Flavins are versatile compounds that are able to function as electrophiles and nucleophiles and sometimes are involved in

catalysis. Riboflavin and its coenzymes have a major role in the detoxification of soil through the hydroxylation of numerous aromatic compounds (Massey, 2000). The oxidation of riboflavin by light, which is a radical reaction, produces lumiflavin and lumichrome. Lumiflavin is predominantly produced in alkali solutions, and lumichrome is predominantly produced in neutral solutions (Fox and Thayer, 1997).

Flavoprotein reducing substrates are mainly dehydrogenated in a two-electron reduction step, and the resulting flavin is re-oxidized by its oxidizing substrate which can be either a two-electron step or a one-electron step. In the one-electron step, the flavin semiquinone is an intermediate (Massey, 2000).

Flavoproteins are able to catalyze a large diversity of reactions. Flavoproteins catalyze the oxidation of α -hydroxyacids and α -amino acids through the dehydrogenation at the α -carbon of the substrate. This results in 2-oxo (α -keto) acid or α -imino acid as primary products (Massey, 2000). The family of proteins known as flavoprotein disulphide reductases contain an active-site disulphide in addition to the FAD prosthetic group. These reactions also involve a pyridine nucleotide as one substrate and a disulphate or dithiol as the other substrate. The enzymes function as part of an oxo acid oxidase multienzyme complex which reoxidizes protein-bound dihydrolipoic acid by reducing NAD to NADH. An example of this reaction would be glutathione reductase utilizing NADPH to reduce oxidized glutathione (Massey, 2000). Flavoprotein mono-oxygenases are another example of enzymes that utilize NADH and NADPH. These enzymes are able to reduce FAD to form a flavin C4a peroxide when the reduced enzyme is reacted with oxygen (Massey, 2000).

Acyl-CoA dehydrogenases are common enzymes that are involved in the oxidation of fatty acids. They oxidize acyl-CoA thioesters to corresponding enoyl-CoA

esters. The reduced flavoprotein that is formed is reoxidized through one-electron transfers to another flavoprotein which is termed the electron-transfer flavoprotein. In mammals, the electron-transfer flavoprotein is oxidized by a third flavoprotein known as electron-transfer flavoprotein-ubiquinone reductase (Massey, 2000).

Riboflavin plays a very important role in antioxidant activity in the body. Although riboflavin itself does not have significant antioxidant activity, it is transformed into FMN and FAD which provide a major protective role over the oxidation of lipid peroxides through the glutathione redox cycle (Rivlin and Pinto, 2001).

Increased riboflavin levels in tissues has been reported to provide protection against oxidative injury caused by oxidized forms of hemeproteins. Studies done by Xu and Hultquist (1991) have shown that dihydroflavin generated by flavin reductase will react rapidly with Fe(IV)O and Fe(V)O oxidation states. The rate of reduction is dramatically increased by riboflavin because it is able to quickly penetrate cells. The riboflavin must be provided exogenously. Another study that was done by Mack et al. (1992) revealed that cardiac damage can be minimized by the administration of riboflavin. The rabbits in this study underwent 60 minutes of hypoxic perfusion while some of them were treated with riboflavin after 55 minutes. Following the hypoxic perfusion, the rabbit hearts were oxygenated for 30 minutes. The results showed that riboflavin reduced cardiac damage when measured as the escape of LDH (Christensen, 1993).

Riboflavin has an effect on the oxidation of amino acids, especially tyrosine, tryptophan, and histidine. Carbon dioxide and an aldehyde are produced by riboflavin-photosensitized oxidation of aliphatic amino acids. Hydroxy radicals and singlet oxygen are responsible for the photooxidation of tryptophan which has electron-rich double

bonds and are excellent reactants for singlet oxygen oxidation. Ascorbic acid can reduce the tryptophan photooxidation because it interacts with excited triplet riboflavin.

Therefore, no riboflavin is available to act on tryptophan (Choe et al., 2005).

Enzymes are also affected by riboflavin photooxidation. The loss of enzymatic activities is common when reactive oxygen species formed by photosensitized riboflavin cause cross-links and protein denaturation in enzymes. Quenchers of singlet oxygen, such as α -tocopherol acetate, β -carotene, sodium azide, and ascorbic acid, can sometimes prevent protein cross-linking and photo-degradation of enzymes (Choe et al., 2005).

Unlike lipids and proteins, carbohydrates are relatively stable when subjected to photooxidation. The photooxidation of glucose can occur when riboflavin is present, but it is dependent on the riboflavin concentration, pH, and glucose concentration. Glucose photooxidation is directly proportional to the concentration of excited riboflavin present (Choe et al., 2005).

Photosensitivity

Riboflavin is light sensitive, especially to UV light, and can generate reactive oxygen species such as superoxide anions and singlet oxygen when exposed to light. This effect not only causes nutrient degradation, but also the off-flavor of foods (Huang et al., 2004a). This is especially a concern for soymilk that is not stored properly because riboflavin can cause significant effects on the head space of oxygen depletion and the formation of volatile compounds when exposed to light (Huang et al., 2004b).

The photolysis of riboflavin in an aqueous solution occurs through 7,8-dimethyl-10-(formylmethyl) isoalloxazine which is commonly known as formylmethylflavin. This is the intermediate which is hydrolyzed to lumichrome and lumiflavin (Ahmad et al.,

2004). The photooxidation of riboflavin is classified into two mechanisms. The first is referred to as type I which involves electron transfer that leads to the generation of the superoxide ion. The second is referred to as type II which involves energy transfer that leads to the formation of singlet O_2 (Silva et al., 1999).

Because the reaction of riboflavin with light can be such a problem, Bhowmik and Sil (2004) examined the effects of reducing agents on retardation of riboflavin oxidation under light. This study found that the visible absorption spectrum of riboflavin in aqueous solution was not affected when different reducing agents were used. There was little change of absorbance and no shift to the absorbance maximum which indicates there was no interaction between riboflavin and the reducing agents. However, the fluorescence spectrum was affected by the reducing agents. The fluorescence of riboflavin was quenched in the presence of the reducing agents which confirms that there was an excited state molecular interaction (Bhowmik and Sil, 2004).

The off-flavor in milk has been contributed to the oxidation properties of the riboflavin present. When the riboflavin in milk is exposed to light, it is transformed into an excited state which can oxidize the methionine in milk to methional which has been confirmed as a component of light induced off-flavor. Photo-degradation of lipids has also been linked to the off-flavor of milk. The degradation of lipids is caused by singlet oxygen oxidizing the fatty acids. The singlet oxygen is found in milk when riboflavin is present (King and Min, 1998; Bender, 2002).

Reactions with other vitamins

Photosensitized oxidation by riboflavin can cause destruction of several vitamins including A, C, D, and E. Vitamin A is destroyed through ring opening, and its loss

increases with the length and intensity of light exposure. This loss decreases with the increase of fat content of milk. The oxidation products produced by vitamin A photooxidation are ethyl-(2,6,6-trimethylcyclohex-1-ene) carboxylate, retinal, 5,8-peroxide of β -ionene, 5,6-peroxide of vitamin A, and retinoic acid. The palmitate ester of vitamin A is also affected by riboflavin oxidation which causes cleavage of its side chain double bonds and creates aldehyde compounds (Choe et al., 2005). Although ascorbic acid is an excellent antioxidant that does not absorb visible light, it is rapidly photooxidized when riboflavin and a high concentration of oxygen are present. The photooxidation of ascorbic acid depends on light intensity, riboflavin, ascorbic acid, and oxygen concentrations, pH, temperature, and the presence of other compounds (Choe et al., 2005). The oxidation of vitamin D is accelerated under light when riboflavin is present via the singlet oxidation pathway, but it only occurs under light and is negligible in the dark (Choe et al., 2005).

There have been studies that confirm riboflavin interactions with other B group vitamins. Due to effects of acute riboflavin deficiency on fetal development being similar to the effects of folate deficiency, there is a possibility that the effects are mediated by flavins on folate metabolism (Powers, 2003). Methionine synthase is an enzyme which converts homocysteine to methionine. It is dependent on 5-methyltetrahydrofolate as a methyl donor but also on vitamin B-12 as methylcobalamin which the synthesis of is dependent on flavoproteins. Even though there is an interaction between vitamin B-12 and riboflavin, there is no substantial proof that riboflavin deficiency will lead to a deficiency in vitamin B-12 (Powers, 2003). There are also similarities between riboflavin deficiency and pyridoxine (vitamin B-6) deficiencies. Riboflavin is required for the conversion of pyridoxine to its coenzyme form pyridoxal

phosphate. Pyridoxine phosphate oxidase, a FMN dependent enzyme, is required.

Those organisms that are deficient in riboflavin will have a reduction in their pyridoxine phosphate which is also required by the body (Laskshmi, 1998). When the riboflavin deficiency was corrected, the activity of erythrocyte pyridoxamine phosphate oxidase increased in human subjects (Powers, 2003; Sauberlich, 1980).

Reaction with Zn

Due to the chemical structure of riboflavin, it has promise for acting as an organic ligand to form metal ion complexes. One of the metal ions that riboflavin can form a complex with is zinc (Zn). The complex formation is able to protect Zn from precipitation into its insoluble form even in biological systems (Agte et al., 1992). FAD shows higher values than riboflavin for complex formation which indicates that the adenine part of FAD may also contribute to complex formation. Intestinal uptake of Zn increases when in a complex with riboflavin or FAD indicating that riboflavin and FAD can act as a carrier for Zn across the intestinal membrane (Agte et al., 1992).

Biosynthesis of riboflavin

Riboflavin is biosynthesized in plants and many bacteria, but humans and most mammals are unable to synthesize riboflavin. Although ruminants cannot synthesize riboflavin, they get the majority of the vitamin from the microflora in their intestines (Bacher et al., 2000). Some bacteria such as the cotton pathogen, *Ashbya gossypii*, can synthesize amounts of riboflavin through fermentation that is well above their metabolic requirement (Bacher et al., 2001; Fischer et al., 2002). This overproduction is beneficial

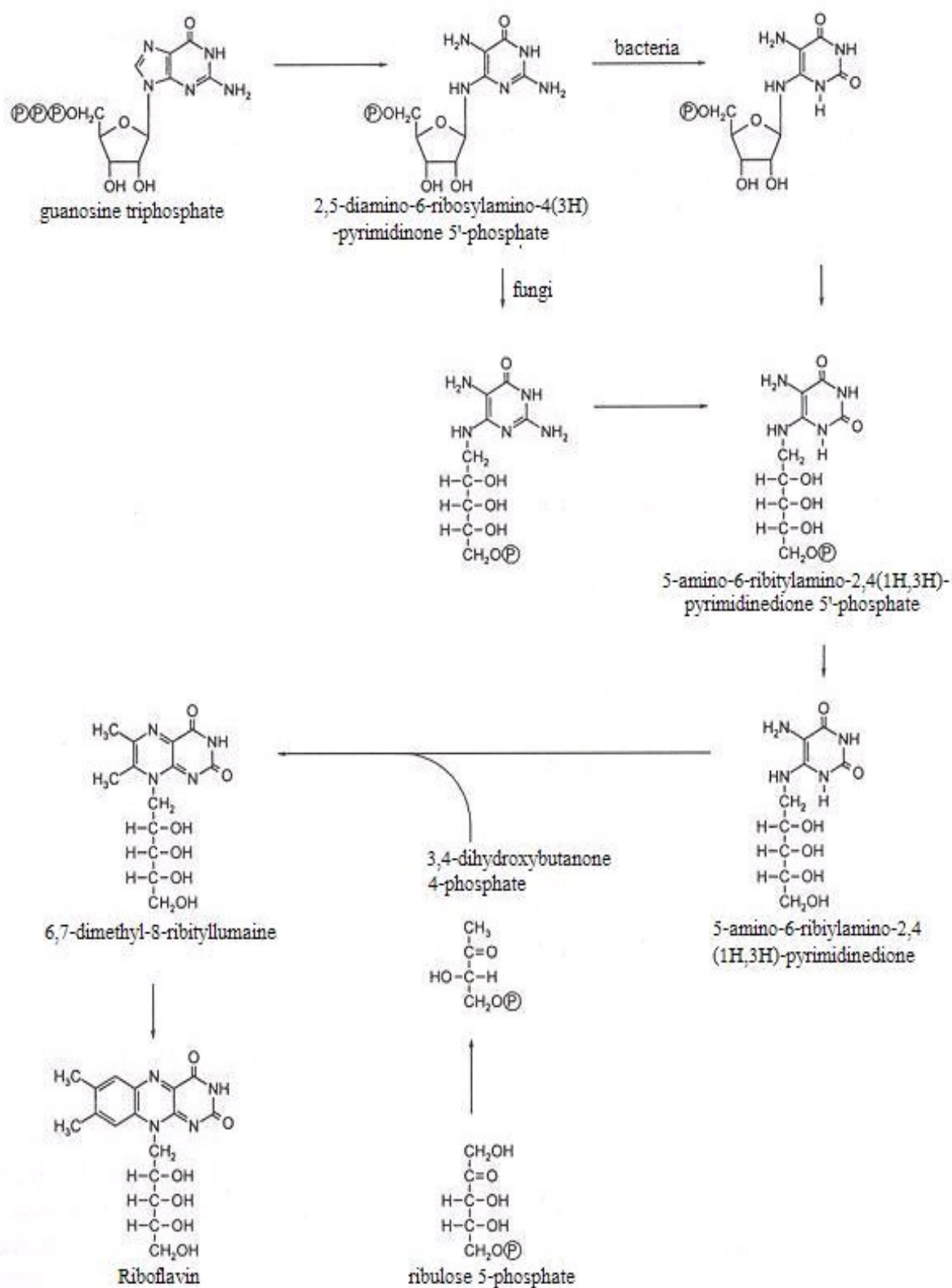


Figure 2.2: Biosynthesis of riboflavin adapted from Bender (2000).

for companies that make commercial riboflavin for laboratory use or vitamin supplements.

Biosynthesis of riboflavin in microorganisms is illustrated in figure 2.2. It is initiated with the hydrolytical opening of the imidazole ring of GTP. This occurs under the release of formate accompanied by the release of pyrophosphate. The pyrophosphate is catalyzed by GTP cyclohydrolase II (Bacher et al., 2000; Fischer et al., 2002). GTP cyclohydrolase II is also the enzyme that is inhibited by the presence of riboflavin when there is an abundance of riboflavin and it does not need to be synthesized (Singhal and Kulkarni, 2004). This reaction produces 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate which is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate using two reaction steps depending on the organism. These two reaction steps involve the hydrolytic cleavage of the position 2 amino group of the heterocyclic ring and the reduction of the ribosyl side chain. This produces the ribityl side chain of the vitamin riboflavin. In eubacteria, the deamination precedes the side chain reduction, while in yeasts and fungi, the reduction precedes the deamination (Bacher et al., 2000).

The 5'-phosphate is dephosphorylated so it can serve as a substrate for 6,7-dimethyl-8-ribityllumazine synthase which condenses the dephosphorylated 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione with 3,4-dihydroxybutanone 4-phosphate (Bacher et al., 2000).

The final biosynthesis step is the dismutation of 6,7-dimethyl-8-ribityllumazine catalyzed by riboflavin synthase which also produces a second product, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione. The entire biosynthesis of riboflavin requires one equivalent of GTP and two equivalents of ribulose 5-phosphate. Only four of the

seventeen riboflavin carbons are not derived from the pentose phosphate pool (Bacher et al., 2000).

Metabolism

Riboflavin plays many key metabolic roles throughout the body. It is important for the production of energy through the coenzyme activity of FAD in the respiratory chain. Many biological reactions that involve oxidation and reduction are mediated by flavin coenzymes. Antioxidant defense is an important function of the body that may be compromised when there is a riboflavin deficiency because the regeneration of glutathione in the glutathione peroxidase antioxidant system requires riboflavin (Villamor et al., 2003).

Approximately 50% of the riboflavin found in plasma is free riboflavin, 44% is FAD, and the remainder is FMN (Bender, 2002). Riboflavin is mostly protein-bound in plasma where free riboflavin binds to both albumin and α - and β -globulins. Riboflavin as well as the coenzymes also bind to immunoglobulins. FMN and FAD that are not bound to proteins are rapidly hydrolyzed to free riboflavin and diffuse into the bloodstream (Bender, 2002).

Absorption

Before absorption of riboflavin derivatives can occur, the vitamin must be hydrolyzed because very little free riboflavin is found in natural sources. This occurs by pyrophosphatase, cleaves FAD and FMN. Alkaline phosphatase, which liberates riboflavin from FMN, releases riboflavin that is ready to be absorbed (McCormick, 1989). Commercial multivitamin preparations are the main source of free riboflavin

(Rivlin and Pinto, 2001). Flavins are absorbed in the upper gastrointestinal tract involving a transport method utilizing a dephosphorylation-rephosphorylation method phosphates present in the intestinal brush border of the upper intestinal tract (Baker, 1995). The action of hydrolases from the brush-boarder membrane, mainly rather than passive diffusion. Transport also involves a saturable ATP-ase active transport system (McCormick, 1990). In the erythrocyte, the riboflavin is trapped metabolically by phosphorylation catalyzed by flavokinase (McCormick, 2003b). After the riboflavin uses active transport to cross into the enterocyte membranes, it is transported to the liver and peripheral tissues in the portal and systemic circulations (Halstead, 2003). An estimate of riboflavin absorption at any one time is 25 mg which is roughly 15 times higher than the RDA. The excess amounts that may be consumed will pass through in the urine and stool (Rivlin and Pinto, 2001). It is generally believed that animal sources of riboflavin are better absorbed than plant sources.

A number of physiological factors have been shown to affect the absorption of riboflavin in the intestines. Certain metals such as copper, zinc, iron, and manganese have been shown to chelate riboflavin and FMN, and inhibit riboflavin absorption (Groff and Gropper, 1999). Diets high in psyllim gum seem to decrease the rate of riboflavin absorbed, but diets high in bran seem to have no effect. Although this diet appears to affect the rate of absorption, there are no indications that it affects the overall amount of riboflavin absorbed (Rivlin and Pinto, 2001). Alcohol, however, affects both the rate of absorption and the amount absorbed. There are indications that recovering alcoholics respond better to vitamin supplements of riboflavin rather than dietary sources (Rivlin and Pinto, 2001). Riboflavin absorption is increased by the presence of food as well as bile salts. The increased absorption with the presence of food may be due to the

decreasing rates of gastric emptying and intestinal transit which prolongs the contact of dietary riboflavin with the absorptive surface of the intestinal cells (Rivlin and Pinto, 2001). Studies have been done with cultured Caco-2 human intestinal epithelial cells which have been helpful for achieving a better understanding of riboflavin absorption. These studies showed that riboflavin uptake is Na^+ and pH independent in these specific cells, but energy and temperature dependent in instances where uptake occurred at 37°C , but this was not the case when uptake occurred at 4°C (Said and Ma, 1994). There is controversy over riboflavin being Na^+ independent because in other studies it has been demonstrated that riboflavin and Na^+ concentrations are linked (Rivlin and Pinto, 2001). These studies also showed that riboflavin was susceptible to the inhibitory effects of sulfahydryl group reagents and amiloride (Said and Ma, 1994).

Different types of cells have a significantly different mechanism for importing riboflavin. The absorption of riboflavin by enterocytes seems to be Na^+ -dependent. The absorption in the proximal small intestine is similar to the absorption of amino acids and glucose. The hepatocytes in the liver exhibit an initial rapid uptake of riboflavin followed by a slower passive diffusion. The riboflavin becomes metabolically trapped in the hepatocytes because it is phosphorylated by flavokinase. The uptake by hepatocytes is relatively insensitive to the Na^+ concentration (McCormick, 1989).

Excretion

Excretion of riboflavin occurs mainly in the urine. Riboflavin undergoes limited metabolism prior to excretion, and mainly free riboflavin is found in the urine (Groff and Gropper, 1999). Unbound riboflavin and riboflavin phosphate that circulate in the plasma are filtered by the glomerous, the phosphate is removed from the riboflavin, and

it is excreted by the kidney (Groff and Gropper, 1999). Riboflavin bound with cysteine and histidine can also be found in the urine if it was absorbed in that form by the gastrointestinal tract or if it is generated in body cells from the degradation of flavoenzymes such as succinate dehydrogenase and monoamine oxidase (Groff and Gropper, 1999). The urinary excretion of riboflavin can be observed a couple of hours after ingestion if the total quantity is greater than 1.7 mg which is similar to what is found in a vitamin pill. The color of the urine will deepen in color to a brighter orangish-yellow when there is an excess of riboflavin (Groff and Gropper, 1999). There are also small amounts of free riboflavin that can be secreted into the bile which is then found in the feces. Fecal riboflavin may also arise from metabolic processes of the intestinal flora (Groff and Gropper, 1999).

Cell functions

The hepatic aryl-hydrocarbon hydroxylase system in the liver is an FAD-dependent P450 cytochromic c enzyme. The cytochromic P450 enzymes are enzymes that shift the absorbance peak of cytochrome chromophore from 420 to 450 nm when it is reduced with carbon monoxide. Many of these P450 enzymes are located in the microsomal fraction where they assist in the metabolism of assorted drugs and toxins as well as being involved in other pathways such as the adrenal steroid biosynthetic pathway (Ross and Hansen, 1992).

The riboflavin carrier protein (RCP) is critical in the reproduction processes of birds and mammals. The vitamin carrier proteins are present in the blood and other bodily fluids, and they ensure that there is optimal bioavailability for growth,

metabolism, and reproduction. These proteins facilitate the dietary absorption, selective transport, and protection against loss by metabolic degradation (Adiga et al., 2002).

The carrier proteins stoichiometrically and reversibly bind vitamins with high affinity and receptor specificity. The riboflavin remains biologically inert while bound to the carrier protein which permits the dissociation of the unmodified vitamin. This allows for optimal utilization at the designated location. The riboflavin carrier protein is induced *de novo* as a reproduction strategy to promote vitamin deposition in the developing oocytes and embryos. The carrier protein sequesters the riboflavin from the maternal circulation and deposits it in the oocyte or embryo against a concentration gradient. Riboflavin has a high fetal to maternal ratio of 4:1 (Adiga et al., 2002).

The mitochondria are an important part of a cell, and riboflavin plays a vital role in the mitochondria's function in the cell. A study was conducted by Barile et al. (2000) to determine the FAD cycle in the mitochondria of rat liver since huge amounts of FAD and FMN are found in the mitochondria. The objective was to determine the process by which the mitochondria provide and regulate their flavin cofactor pool. The riboflavin and FAD found in the mitochondria are precursors for the synthesis of mitochondrial flavin cofactors essential for the normal functioning of the mitochondria. The riboflavin is transported into the mitochondria via carrier proteins, and FAD is synthesized by FAD synthetase which is localized in the matrix. The matrix has a substrate pair of mitochondrial ATP and FMN synthesized from riboflavin through the putative mitochondrial riboflavin kinase. The synthesis of FMN and FAD occurs in the cytosol where the enzymes riboflavin kinase and FAD synthetase exist. The mitochondria can synthesize FAD from the FMN which is taken up through carrier proteins. Because mitochondria possess FAD pyrophosphatase and FMN phosphohydrolase, FAD and

FMN can be catalyzed to produce riboflavin in the intermembrane space (Barile et al., 2000). Riboflavin is important in the mitochondria and peroxisomes because this is where the β -oxidation of fatty acids occurs. The mitochondrial pathway requires the import of a long chain fatty acyl-CoA which is acted upon by an FAD-dependent acyl-CoA dehydrogenase. This reaction produces an α - β unsaturated acyl-CoA and FADH_2 . The electrons produced from the FADH_2 as well as NADH are introduced into the electron transport chain to generate two and three ATP molecules, respectively (Ross and Hansen, 1992).

Elevated homocysteine concentrations in the blood have been associated with an increased risk for cardiovascular disease and decreased cognitive function.

Homocysteine metabolism is regulated by various enzymes and B-vitamins including riboflavin. Methylene tetrahydrofolate is a coenzyme of riboflavin, and it catalyzes N^5 - N^{10} methylene tetrahydrofolate to N^5 -methyl tetrahydrofolate which is required for remethylation of homocysteine to methionine. Low blood concentrations of B-vitamins that are involved in the methionine metabolism can lead to elevated levels of homocystein (Ganji and Kafai, 2004; Lin et al., 2004).

Riboflavin Deficiencies

Although riboflavin deficiencies are not commonly seen in the United States, it is a significant public health problem in other areas of the world such as those populations who's main staple is rice (Laskshmi, 1998). Fortunately, riboflavin deficiencies are rarely, if ever, fatal due to the efficient conservation and reutilization of tissue riboflavin when there is a dietary deficiency. The only riboflavin that will be lost during a deficiency is the small amount that is covalently bound to enzymes (Bender, 2002).

Riboflavin deficiency may be acute or chronic. The acute deficiency is caused by borate poisoning which replaces riboflavin in binding sites, forms a complex with the ribityl side chain, and increases loss of the vitamin to the urine. Chronic riboflavin deficiency is unknown as an endemic disease because of the widespread consumption of milk and cheese. It is, however, a problem in many areas of the world where the population subsists on unenriched cereal diets (Roe, 1991). Riboflavin is widespread throughout many different foods and diets, so most diets will provide a minimal amount of riboflavin to permit maintenance of central metabolic pathways (Bender, 2002). In the liver, there is only a four-fold difference between deficiency and saturation, and in the central nervous system, there is only a 35% difference between deficiency and saturation (Bender, 2002). Riboflavin deficiency usually does not occur by itself, but is more often associated with other water-soluble vitamin deficiencies (Eitenmiller and Landen, 2000).

Riboflavin deficiency is usually distinguished by lesions of the margin of the lips and the corners of the mouth, desquamation of the tongue, seborrheic dermatitis, filiform excrescences affecting the nasolabial folds, eyelids, and ears, and abnormalities of the skin around the vulva, anus, and prepuce (Bender, 2002). Deficiencies can also cause conjunctivitis with vascularization of the cornea and opacity of the lens. This is the only part of the disease where the biochemical cause is known. Glutathione reductase is a flavoprotein that is particularly sensitive to riboflavin depletion, and glutathione is important in maintaining the normal clarity of the crystalline in the lens of the eye (Bender, 2002). Although riboflavin is required for the proper functioning of the eye, it can also be damaging. The vitamin C that is present at high concentrations in normal lenses plays a very important role in inhibiting the photosensitization process that can occur to riboflavin in the eye (de La Rochette et al., 2000).

Factors other than sub-optimal intake can cause a riboflavin deficiency as well. Adrenal insufficiency and interactions with alcohol or other drugs such as psychotropic, chemotherapeutic, and antimalarial medications can cause a deficiency in riboflavin. Some of these drugs include chloromazine, imipramine, amitriptyline, doxorubicin, and quinacrine (Villamor et al., 2003 and Rucker, 2000). These drugs have structures that resemble riboflavin and act as antagonists that inhibit the conversion of riboflavin to its coenzyme forms by inhibiting flavokinase activity. The administration of adriamycin which is an anti-cancer drug also inhibits FAD biosynthesis (Laskshmi, 1998).

Glutathione reductase activity is important for red blood cells to effectively regulate cellular redox potential through glutathione peroxidase. When there is an insufficient amount of riboflavin in the diet, studies in animals have shown there is an increased potential of the cell to sustain oxidative injury causing hemolytic anemia (Harmatz et al., 2003).

The main metabolic effect of riboflavin deficiency is on lipid metabolism which causes a lower metabolic rate (Bender, 2002). Study animals require 15-20% more feed to maintain the same weight as that of control animals, and feeding a diet high in fat to riboflavin deficient animals reveals a more differentiated impairment of growth (Bender, 2002).

Although a report of personality changes in riboflavin deficiency have not been substantiated, riboflavin plays a role in thyroxine metabolism and a deficiency may contribute to the pathophysiology of some mental illness (Powers, 2003). Symptoms of neurodegeneration and peripheral neuropathy have been observed and documented in studies of riboflavin deficiency in young, rapidly growing chickens, racing pigeons, and rats. There is little information about this condition with humans, but there is a

documented case of a 2.5 year old girl who was riboflavin deficient and exhibited neurological abnormalities such as anemia and visual impairment (Powers, 2003).

Some studies demonstrate that there were both increases of cancer and possible protection from cancer with the deficiency of riboflavin (Powers, 2003). Because some carcinogens are metabolized by flavin-dependent enzymes, riboflavin deficiency in these cases may enhance or diminish the effect of the carcinogen. Recently, one work showed there was a connection between deficiency of riboflavin and an increase in DNA breakage in rats when exposed to hepatocarcinogens. Because of the DNA breakage, there was induction of the repair enzymes which help resistance to malignant transformations to be enhanced in the deficient animals (Powers, 2003).

Animals can also exhibit signs of deficiency. Some symptoms are similar to those seen in human patients. The most common signs of deficiency are cessation of growth, dermatitis, hyperkeratosis, slopecia, and vascularization of the cornea (Trusswell and Milne, 1998). In the fetus, abortion or skeletal malformations are possible. Also, in some species, anemia, fatty liver, and neurological symptoms have been observed (Trusswell and Milne, 1998). A problem found in equines is uveitis which is also known as moonblindness and can occur from but is not limited to a riboflavin deficiency. This disease affects the horses night vision which is normally relatively good (Goff, 2004).

Riboflavin deficiencies have an effect on the body's normal handling of Fe. It impairs absorption as well as increases the loss of gastrointestinal endogenous Fe. The hyperproliferation of crypt cells and an increased rate of transit of enterocytes along the villi seem to cause the effects observed with absorption and gastrointestinal loss (Powers, 1995). The problem seen with the crypt cells and enterocytes along the villi probably lead to the development of functionally immature villi which reduces the

absorptive surface area for Fe. The Fe loss was also linked to the high turnover rate of the epithelial cells of the small intestine when there was a riboflavin deficiency (Powers, 1995).

Studies in geographical areas of the world that have a high rate of malaria have shown that riboflavin-deficient human subjects are relatively resistant and have a lower parasite burden than those subjects that are adequately nourished (Bender, 2002; Rivlin and Pinto, 2001). Although the biochemical basis of why this occurs is unknown, there are two proposed mechanisms. First the malarial parasites require a relatively high level of riboflavin. A decrease in riboflavin impairs the growth of parasites because they are not getting the required amounts of the vitamin. Secondly, because of an impaired antioxidant activity in the erythrocytes, the erythrocyte membrane might be more fragile or have reduced membrane fluidity. This may expose the parasites to the host's immune system at a more vulnerable stage which results in the production of antibodies. This is similar to the sickle cell trait which also protects against malaria (Bender, 2002; Rivlin and Pinto, 2001; Bhaskaram, 2002).

Although there is thought to be an effect of riboflavin deficiency on physical performance, there are very few studies that actually support this. It is believed that in countries where there is no evidence of malnutrition that the riboflavin status is different between athletes and nonathletes. There is also no evidence that riboflavin supplements increase performance in healthy, well nourished individuals (Powers, 2003). A study on women showed that, while aerobic exercise increased the riboflavin requirements, additional riboflavin intake improved endurance. However, pregnant women, who participated in a walking program and took vitamin-mineral supplements containing riboflavin, slightly improved their aerobic capacity (Perelson and Ellenbogen, 2002).

Riboflavin deficiencies can be treated with oral doses of riboflavin up to 25 mg. A single dose greater than this cannot be completely absorbed by the body even though it is safe to administer. The absorption of the administration dose is limited by the low solubility of riboflavin (Rivlin, 2002) and the storage capacity (Rivlin and Pinto, 2001). Animal studies have shown an upper limit to tissue storage of flavins that cannot be exceeded under normal circumstances. The storage capacity of the tissues is presumably limited by the availability of proteins that provide binding sites for the flavins (Rivlin and Pinto, 2001). Although consumption of mega-doses of riboflavin have not been shown to be toxic, there is a theoretical risk for the administration of riboflavin. It has photosensitizing properties which may result in DNA degradation and an increase in the formation of lipid peroxides (Misra et al., 2001; Rivlin, 2002). When riboflavin forms an adduct with tryptophan, the photodegradation is accelerated. Administration of riboflavin to the skin may increase the synthesis of melanin because of the stimulation of free-radicals which increases the risk of skin cancer (Rivlin and Pinto, 2001).

There has been experimentation in people with riboflavin sustained release floating capsules for the treatment of riboflavin deficiencies. The theory behind these capsules is that riboflavin is not always readily absorbed because of the fast passage rate (El-Samaligy et al., 2003). The capsules are prepared with a component such as sodium bicarbonate which is liberated by the CO₂ content of the stomach and rises to the top of the content of the stomach. By having the riboflavin pass through towards the end of the digestion, the passage rate is slowed to provide more time for riboflavin to be in contact with the small intestine (El-Samaligy et al., 2003). This study showed an increase of riboflavin concentration in the body using the capsules that caused the vitamin to float to

the top compared to the capsules that did not cause the vitamin to float to the top (El-Samaligy et al., 2003).

Riboflavin Detection

Fluorimetry

The AOAC methods for direct fluorimetric detection of riboflavin are based on the conversion of FAD and FMN to riboflavin giving a measurement of the total riboflavin in the sample. Direct fluorimetry quantitates riboflavin using fluorescence measurements that use an input filter of narrow T range with a wavelength maximum of 440 nm and a narrow T output range with a wavelength maximum of 565 nm. To prepare the sample for this procedure and reduce any background fluorescence, the sample is acidified with glacial acetic acid and oxidized with potassium permanganate. After two minutes, the excess potassium permanganate is destroyed with hydrogen peroxide (Eitenmiller and Landen, 2000).

Indirect fluorimetry can also be used to determine riboflavin concentration. This method utilizes the lumiflavin to quantitatively determine the amount of riboflavin. The lumiflavin is formed when the sample is irradiated under alkaline conditions. It fluoresces more strongly than free riboflavin, and it is chloroform soluble which provides a means of removing interfering fluorescent compounds from the sample matrix. This procedure is more sensitive than direct fluorimetry of riboflavin due to the increased fluorescence of lumiflavin. Although this procedure is not commonly used in the United States, it is able to eliminate sensitivity problems and poor chromatograms that are associated with the direct analysis of riboflavin (Eitenmiller et al., 2000). Both

of the fluorometric methods have some documented weaknesses. They have a tendency to overestimate the total riboflavin content which is due to the interference from fluorescing impurities from the sample matrix (Russell and Vanderslice, 1992).

HPLC

HPLC, high performance liquid chromatography, has many advantages. It can be developed for almost any pharmaceutical or biological matrix, it is sensitive, specific, fast (Eitenmiller and Landen, 2000), and can assay multiple vitamins simultaneously (Russell, 2000). The drawbacks of this assay is that it requires expensive equipment and trained personnel. In addition, the determination of naturally occurring vitamins is somewhat limited and it cannot be guaranteed that it is able to account for all the active forms of a vitamin that would normally be quantified in a microbial assay (Ball, 1994). HPLC may also underestimate vitamin content (Ball, 1994). Extraction from the food matrix is usually the greatest challenge for vitamin analysis, especially when the vitamins are often bound to other food constituents such as carbohydrates and proteins (Russell, 2000).

For HPLC detection, ultraviolet absorbance is probably the most common method followed by fluorescence and electrochemical detection. The fluorescence and electrochemical detection are generally used when physiochemical properties permit and increased sensitivity and selectivity are desired. Refractive index can also be used, but it is not a common procedure because it lacks specificity and sensitivity (Russell and Vanderslice, 2000).

An interlaboratory comparison of HPLC and microbiological methods for total riboflavin revealed significant variability between the 13 labs that participated. The

most likely source of the of the variability seems to be the extraction and hydrolysis methods used by the different laboratories (Russell, 2000; van den Berg et al., 1996).

Immunoassays

Immunoassays and protein-binding assays are also possible for the quantification of vitamins. These assays are as sensitive as the other methods and are less prone to interference. Large numbers of samples can be run using ELISAs and non-isotopic protein-binding assays that utilize microtiter plates. These assays do not have the problem of handling and disposing of radioactive waste (Ball, 1994). The immunoassays and protein-binding assays chemically or enzymatically extract the vitamins from the food matrix to ensure the riboflavin is in its free form before being run on the microtiter plate. The use of these assays depends on the successfulness, applicability, and validation as well as the commercial availability of standardized kits (Ball, 1994).

Microbiological assays

Test organisms for vitamin assays must specifically require the selected organism of interest to be genetically constant during prolonged response, have a rapid growth cycle, have a growth response that is not easily influenced by neutralization salts or other substances that may be present in a sample extract, and be nonpathogenic (Voigt and Eitenmiller, 1985).

A recent method developed for the determination of riboflavin in samples involves agar-diffusion. This is able to measure riboflavin concentrations in biological samples using the bacterium *Bacillus cereus*. The studies conducted found this assay to be highly reproducible, sensitive, rapid, inexpensive, and can be applied to measure the

amount of riboflavin in desired samples. This assay is considered a good candidate for the screening of bacteria growing on media that secrete riboflavin (Salveti et al., 2003).

The classical way to determine riboflavin concentrations in samples is to use the microbiological method using *Lactobacillus rhamnosus* ATCC 7469. The assay is almost universally used and applicable to most matrices. The microbial assay has equipment, bacterial cultures, and ready-made media commercially available, and the assay procedure for riboflavin is standardized (Ball, 1994). Microbial assays have the advantage of space, labor, materials, and time over biological methods using animals (Voigt and Eitenmiller, 1985). Vitamin determination using microbiological methods is based on the nutritional requirement of the specified organism for the vitamin of interest. A basal medium provides all of the growth requirements for the organism except the vitamin being assayed. A growth response curve can be generated using a defined concentration range. The growth response is directly proportional to the amount of vitamin added to the medium. To determine the amount of vitamin present in the sample, the growth can be measured photometrically or metabolic products can be monitored (Voigt and Eitenmiller, 1985).

Organisms other than *L. rhamnosus* that have riboflavin requirements are *Leuconostoc mesenteroides* and a protozoan *Tetrahymena pyriformis*, but these organisms have not been used much for riboflavin assays. *T. pyriformis* requires 4-5 days to achieve a stable growth response. This is a longer time than that of *L. rhamnosus* which takes 16-18 hours. *T. pyriformis* is also more sensitive to matrix interference and to citrate ions that can be present in food extracts than *L. rhamnosus* (Eitenmiller and Landen, 2000).

Because FMN and FAD have very different growth-promoting effects and *L. rhamnosus*' response to free riboflavin is significantly greater than that of its coenzymes, the FMN and FAD in the sample have to be converted to free riboflavin. This is achieved by acid hydrolysis. The AOAC International extraction procedure (Chapter 45.2.06, Method 940.33) is the most common method used for riboflavin determination. It uses hydrolysis with 0.1 N HCl at 121 °C for 15 minutes to treat the sample. The filtrate pH is adjusted to 6.8 with sodium hydroxide prior to incorporation into the assay. This acid hydrolysis sufficiently converts FAD and FMN to free riboflavin for utilization by *L. rhamnosus* (Eitenmiller and Landen, 2000). The drawback of this procedure is the cumbersome nature, long incubation times, tedium, and poor precision (Ball, 1994).

Lactobacillus rhamnosus

To do a microbiological assay on a specific nutrient, a bacterium or yeast that is an auxotroph is needed. This means that the organism being used to detect the desired nutrient requires the nutrient, but is not able to synthesize it. For the riboflavin microbiological assay, the bacterium of choice is *Lactobacillus rhamnosus* which was formerly known as *Lactobacillus casei* subspecies *rhamnosus* ATCC 7469. It requires riboflavin for growth, but it is not able to synthesize it on its own which makes it a good candidate to use for the quantitative determination of riboflavin in samples (Angyal, 1995).

Lactobacillus rhamnosus is a gram positive, non-spore forming rod that grows best between 15 °C and 45 °C. This bacterium is also microaerophilic which indicates that growth on solid media is usually enhanced by anaerobiosis and 5-10% CO₂ (Sneath et al., 1986). The pH requirements for this organism are slightly acidic and optimal

between 5.5 and 6.2 for growth, but growth ceases when a pH of 4.0-3.6 is reached. The growth rate is often reduced at a neutral or alkaline pH (Sneath et al., 1986).

This bacterium is often found in food products such as dairy, grain, meat, fish, water, beer, wine, fruits, pickled vegetables, sauerkraut, and sour dough. They can also be found in sewage, silage, and mash as well as the natural flora in the mouth and intestinal tract of many homothermic mammals including humans. Pathogenicity of this organism is extremely rare (Sneath et al., 1986).

Rationale and Significance

Riboflavin is an essential part of the diet because it cannot be synthesized by many mammals (Bacher et al., 2000). Because this compound is a required, it is considered a vitamin and is also known as vitamin B2 (Carpenter, 2003). Detection and determination of riboflavin in food and animal feed samples is important to ensure that the minimum amount of riboflavin required for normal growth is being obtained in the diet. The human population does not generally have a problem with meeting the minimum riboflavin requirement if a balanced diet is consumed. Certain parts of the world in which the main food source is rice is found to have a more significant riboflavin deficiency problem (Laskshmi, 1998). There can also be a problem when food is processed before consumption because this can have an effect on the riboflavin content. The factors that can affect riboflavin concentration in food products are heat, moisture, oxygen, pH, and light. These factors can be encountered through milling, fermentation, germination, extrusion, and thermal processing (Reddy and Love, 1999). Because so many different factors and processes can affect riboflavin and because many of the foods consumed are processed, quantification of vitamin concentration in foodstuffs can be

important to prevent riboflavin deficiencies in certain populations. Quantification of riboflavin is also important for the cereal grain products that are often fortified with riboflavin to ensure people are receiving the recommended daily allowance (McNulty et al., 1996). Deficiencies can include lesions of the lips, desquamation of the tongue, and abnormalities of skin around the vulva, anus, and prepuce (Bender, 2002).

Microbiological assays are a common way of determining vitamin concentrations in food samples. These assays have the advantage of equipment, ready-made media commercially available, space, labor, materials, and time (Voigt and Eitenmiller, 1985). The riboflavin assay has been standardized for use with test tubes by AOAC (Ball, 1994), but this assay has the capability of being more user friendly by adaptation to microtiter plates. The microbiological folic acid assay also uses the same bacterium, *L. rhamnosus* ATCC 7469, and this assay was successfully scaled down to microtiter plates (Horne, 1997). The microtiter plate assay was able to save time, labor, and supplies and was able to assay more samples at one time (Horne, 1997). The adaptation to microtiter plates for the riboflavin assay would produce the same advantages as that of the folic acid assay.

CHAPTER III

MICROTITER PLATE ADAPTATION OF RIBOFLAVIN TUBE ASSAY

Synopsis

Riboflavin is an essential part of the diet by playing a significant role in the metabolism of carbohydrates, fatty acids, and amino acids. Many cereal-grain products are currently being fortified with riboflavin to reduce the possibility of deficiency in diets that are not normally sufficient in riboflavin. The objective of this research was to reduce time of the assay by scaling it down to microtiter plates while still maintaining the precision of the original tube assay. This scaled-down assay was utilized for the determination of riboflavin in samples of commercial maize, commercial ground soybean meal, and commercial breakfast cereals. The organism that is used most commonly to quantitate riboflavin is *Lactobacillus rhamnosus* ATCC 7469. The AOAC approved riboflavin assay was used as a guideline for transferring the riboflavin assay from a tube assay to a microtiter plate assay. Riboflavin assay *L. rhamnosus* was grown overnight at 37°C for 8 hours and absorbances were read on a microtiter plate reader. The standard growth curve for the riboflavin assay was linear from 0 ng/mL up to 10 ng/mL with an average R^2 value of 0.99. The data showed no significant difference between the tube assay and microtiter plate assay ($P < 0.05$) for the commercial maize sample. Other samples were used to confirm reproducibility. The microtiter assay reduced the amount of time required for sufficient bacterial growth response to generate linear standard curves from 22 hours to 14 hours.

Introduction

Riboflavin is an important requirement for most organisms. Some organisms are able to synthesize riboflavin while other organisms that are higher up in the food chain cannot and must acquire riboflavin from their diet (McCormick, 1989). Riboflavin can be consumed in three forms which include free riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). The last two represent the phosphorylated forms (Powers, 1999). Although all three forms are found intracellularly, absorption occurs as riboflavin which is followed by intracellular phosphorylation in the enterocytes. FAD is the most common form of riboflavin present in tissues and in circulation (Powers, 1999). The coenzyme forms of riboflavin, FMN and FAD, can serve as prosthetic groups of many holoenzymes. These holoenzymes can catalyze essential one and two electron oxidation-reduction reactions (McCormick, 1989).

Microbiological quantification of vitamins requires a microorganism that has a need for a certain vitamin without being able to synthesize the vitamin being studied. This process allows for the use of a medium that contains all of the growth requirements for the microorganism being used except the vitamin being assayed (Angyal, 1995). The vitamin of interest can be added to the media in certain concentrations to obtain a growth curve for a specified amount of time. A sample with an unknown concentration of the vitamin of interest can also be determined by comparing the microorganism growth of the sample with that of the standard curve (Voigt and Eitenmiller, 1985). The riboflavin assay has already been tested and proven to work in tubes using *L. rhamnosus* as the microorganism (Angyal, 1995).

In the food processing industry, large numbers of samples need to be processed quickly. A rapid assay was developed for folic acid by adapting the tube assay to microtiter plates (Horne, 1997). The goal for developing the riboflavin assay was to decrease the total amount of time required for the assay and make it easier to run more samples at one time while retaining the accuracy and precision of the tube assay. Microtiter plates provide many benefits when compared to tube assays. The development of a microtiter plate assay can save commercial industry time, space, material, and labor (Newman and Tsai, 1985). Time can also be saved because many more trials can be prepared and conducted simultaneously since microtiter plates do not require as much space or media as tubes (Voigt and Eitenmiller, 1985).

Because *L. rhamnosus* requires riboflavin to grow, has a rapid growth cycle, is not easily influenced by substances that are present in sample, and is nonpathogenic, it is a common organism to use for the quantification of riboflavin in samples (Voigt and Eitenmiller, 1985). *L. rhamnosus* is a nonsporing, gram positive rod bacterium. It is facultatively anaerobic, and when grown under anaerobic conditions, the fermentation products produced are mainly lactate, but it may also give acetate, ethanol, and CO₂ (Sneath et al., 1986). *L. rhamnosus* is an obvious choice for studying riboflavin content because it is simple to work with and is not a human pathogen (Sneath et al., 1986). This paper describes a microtiter assay for measuring riboflavin concentrations in samples using a riboflavin auxotroph, *L. rhamnosus*, which is used in the standardized tube riboflavin assay.

Materials and Methods

Bacterial strain

L. rhamnosus American Type Culture Collection (ATCC, Manassas, VA, USA) #7469 was the organism used in the riboflavin assays. Stock cultures of *L. rhamnosus* were transferred monthly into stab cultures consisting of 10 mL of Micro Assay Culture Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). They were incubated at 37°C for 24-48 hours and transferred to a refrigerator held at 4°C for storage. A working culture was kept on a streak plate and was transferred weekly. The streak plate was stored at 4°C.

To prepare the inoculum for the riboflavin assay, a subculture of the *L. rhamnosus* stock culture was made by inoculating 10 mL of Micro Inoculum Broth. The broth was incubated for 16-24 hours at 37°C. The culture was centrifuged using aseptic technique, and the liquid was decanted. The cells were washed three times with 10 mL of 0.85% sterile saline solution, and they were resuspended in 10 mL of 0.85% sterile saline solution. Using a spectrophotometer at 600 nm, the absorbance of the cell suspension was adjusted to 35-40% transmittance for tubes and 10-15% transmittance for microtiter plates using sterile saline solution to dilute the cell suspension. This cell suspension, 20 µL for tubes and 4 µL for microtiter plates, was used to inoculate broth for the assay. Both the tubes and the microtiter plates were kept at 37°C.

Media preparations

The media was prepared by suspending 4.8 grams of Bacto® Riboflavin Assay Medium (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) in 100 mL of

deionized water. The media was boiled 2-3 minutes to dissolve completely. It was then dispensed in 5 mL amounts into tubes. The standard or test sample was added, and the volume was adjusted to 10 mL using deionized water. The tubes were autoclaved for 10 minutes at 121 °C.

Standard curve preparation

The riboflavin stock solution was prepared by weighing 50 mg of riboflavin standard (Sigma) and dissolving it in 500 mL of deionized water in a volumetric flask. Then, it was further diluted by adding 0.5 mL to 500 mL of deionized water in a volumetric flask. The diluted stock solution was distributed in the tubes using 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0 mL per tube. These volumes were adjusted to 10 mL with 5 mL of media and deionized water which gave riboflavin concentrations of 0.0, 2.5, 5.0, 7.5, 10, 15, 20, and 30 ng/mL. The tubes were prepared in duplicate. The stock solution for the standard curve was prepared fresh daily to prevent deterioration of riboflavin in the solution.

Culture growth conditions

Cell growth was measured turbidimetrically at 600 nm (A_{600}) on a Milton Roy Spectronic 20D spectrophotometer for the tubes and a Tecan Spectra Fluor Plus (Research Triangle Park, NC) for the microtiter plates. The tube assays were allowed to grow for 18-24 hours as recommended by the commercial supplier (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) while the microtiter plates were allowed to incubate for 14-22 hours. The cells exhibited a lag phase growth period which is defined

by the time period between the initiation of growth on the growth curve and exponential phase of the growth curve.

Sample preparation

Samples were weighed to 0.500g +/- 0.01g and transferred to an anaerobic culture tube. To each sample, 12.5 mL of 0.1 M sulfuric acid was added, and a crimped top was added to the tube. The samples were autoclaved for 15 minutes along with the prepared riboflavin standard tubes. After removing the tubes from the autoclave and cooling, they were vortexed and the pH was adjusted to 6.8 using 0.37 N sodium hydroxide. The volume was then adjusted to a final volume of 25 mL with deionized water. The samples were poured into 50 mL centrifuge tubes and centrifuged for 10 minutes at 5000 rpm. The samples were filtered with a 0.2 μ m syringe filter (Whatman, Clifton, NJ, USA) into a sterile 50 mL centrifuge tube. They were disbursed into tubes containing 5 mL of Bacto[®] Riboflavin Assay Medium (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) and deionized water, and were autoclaved along with the prepared riboflavin standard tubes. The final tube volume was 10 mL after the sample was added. The samples were kept either in tubes for the tube assay or transferred to microtiter plates. They were inoculated with the same amount of bacterial solution as the standard curve and subjected to the same growth conditions as the respective riboflavin standard.

Nalidixic acid preparation

Nalidixic acid was added to deionized water to generate a stock solution which was filtered through a 0.2 μ m syringe filter. Nalidixic acid was added to the sample

tubes after they were autoclaved and cooled. The final concentration of nalidixic acid in the sample tubes was 20 $\mu\text{g/mL}$ per tube.

Statistical analysis

The data generated using the microbiological riboflavin assays were analyzed using the Duncan's test and regression analysis. All statistical analysis was considered significant at $P < 0.05$. The statistical data were generated using Statistical Analysis Software 8.0 (SAS) (SAS Institute, Cary, NC, USA).

Results and Discussion

Nalidixic acid

Nalidixic acid was used in this study to decrease the possibility of contamination of media. This antibiotic was selected for use after screening *L. rhamnosus* with a range of antibiotics that included tetracycline, erythromycin, neomycin, nalidixic acid, and kanamycin. The only antibiotic that *L. rhamnosus* showed a natural resistance to was nalidixic acid. To ensure that nalidixic acid did not retard the growth of *L. rhamnosus*, standard curves with nalidixic acid were compared with those that did not have the antibiotic. The results of these studies are depicted in figure 3.1. The nalidixic acid was found to have no effect on the growth of *L. rhamnosus* for the riboflavin assay.

Microtiter plate modifications

By 1940, remarkable achievements were being made with bacterial assays. This work permitted researchers to make progress in one year that would have otherwise

Comparison of Standard Curve and Standard Curve plus Nalidixic Acid

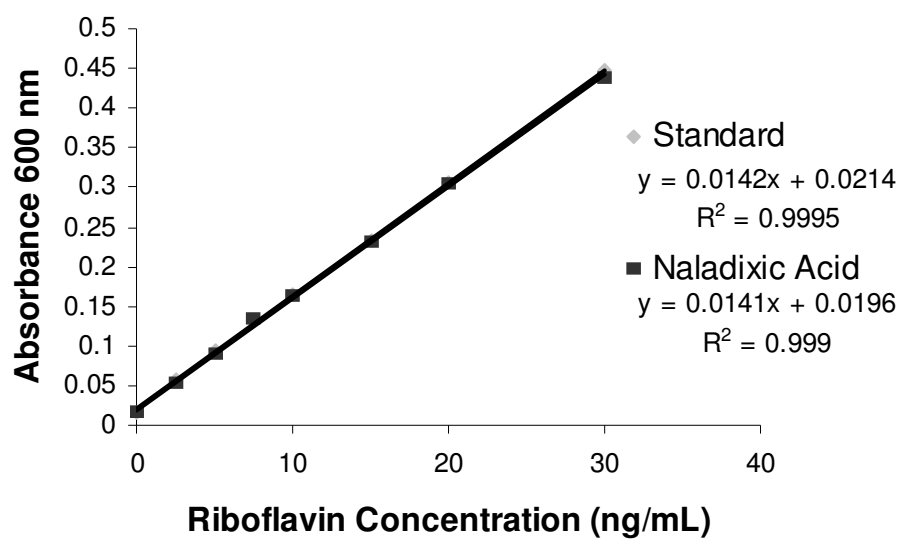


Figure 3.1: Comparison of microtiter plate riboflavin standard curve with the standard curve plus nalidixic acid.

taken ten or more years (Kavanagh, 1963). Further modification of the bacterial assays to microtiter plates can save even more time. Microtiter plates have been successfully developed for the detection of bacterial growth to quantify organisms via most probable number (Hernandez, 1991). Microtiter plates have also been used to modify the folic acid tube assay using *L. rhamnosus*. This assay improved the analysis by allowing more samples per day with a sensitivity comparable to that of the tube assay (Newman and Tsai, 1985; Horne, 1997). The microtiter scaling of the folic acid assay proved to reduce culture volumes by 10-fold compared with other suspension culture microbiological assays. By decreasing culture volumes, the cost of reagents and minimum amount of folate measurable was proportionately lower (Newman and Tsai, 1985). The modifications made to the folate assay using microtiter plates retained the sensitivity and selectivity of procedures previously done to detect folate (Newman and Tsai, 1985). The growth rate of the riboflavin assay was determined for tubes as well as microtiter plates. Microbial growth in microtiter plates reached its maximum approximately 4 hours before the tube assay reached its maximum growth (figure 3.2). This allows for more rapid results with the microtiter plate assay versus the tube assay.

To test the suitability of the microtiter plate approach for assaying riboflavin concentrations in samples, riboflavin concentration was determined in a commercial maize sample and used to compare the microtiter plate assay with the standardized tube assay. Each microtiter plate assay was run simultaneously with a tube assay for comparison. A standard curve was prepared each time a trial was run. It was important to construct a standard curve each time the assay was run because of fluctuations in autoclaving conditions, incubation temperature, and other environmental conditions that cannot be controlled. Slight differences in these variables could influence the standard

curve readings in each trial. The riboflavin concentration in the commercial maize sample was determined by using the absorbance at 600 nm for each sample and putting it into the equation that was generated from the standard curve for each trial. This number was then converted into milligrams of riboflavin per kilogram of sample for ease of comparison between samples.

Sample results

The reproducibility of results is important for quantification of riboflavin in the assay. The six riboflavin tube assay trials verified that there was no significant difference between any one trial (figure 3.3). Six riboflavin microtiter plate assay trials also verified that there was no significant difference between any one trial (figure 3.4). It is also important to determine if the developed microtiter plate assay is as accurate and compared to the tube assay(figure 3.5). This figure shows no significant differences between the microtiter plate assay and the tube assay for the determination of riboflavin concentration.

Other samples were also used to determine the reproducibility of the riboflavin microtiter plate assay. These samples included ground soybean meal, commercial oatmeal, commercial cornflakes breakfast cereal, commercial rice squares breakfast cereal, and commercial wheat squares breakfast cereal. They were chosen because many cereals are fortified with riboflavin to enhance a balanced diet, and they were available commercially. These samples were treated with acid and autoclaved to get an accurate determination of riboflavin content. Acid hydrolysis of the samples is important for the conversion of biological forms of riboflavin known as flavin mononucleotide (FMN) and

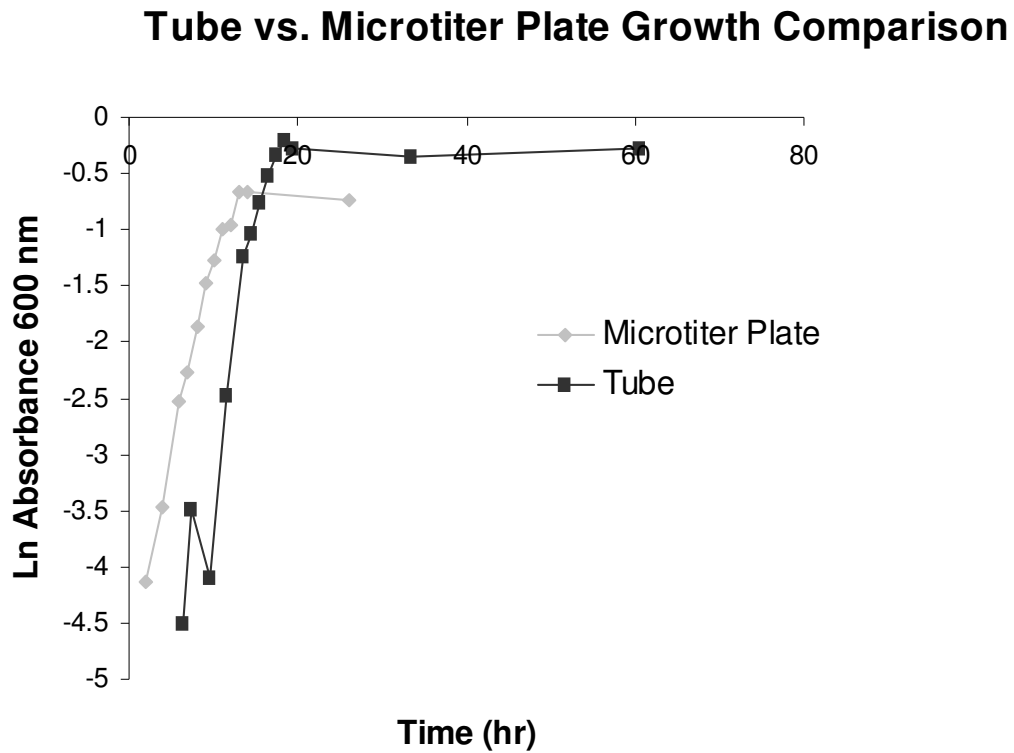


Figure 3.2: Comparison of growth rates for riboflavin tube assay and riboflavin microtiter plate assay. There were three tubes per trial ($n = 3$) and six trials for each point. For the microtiter plate there were eight wells per trial ($n = 8$) and six trials for each point.

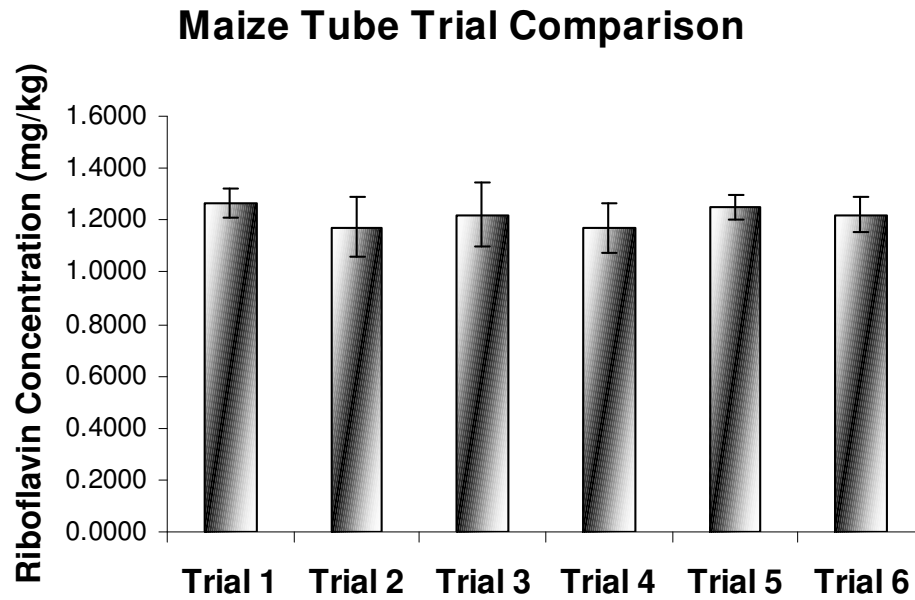


Figure 3.3: Shows the consistency between trials for the riboflavin tube assay using commercial source of maize as a sample using the standard deviation. There was no significant difference between trials ($P < 0.05$). Each trial had three replicates.

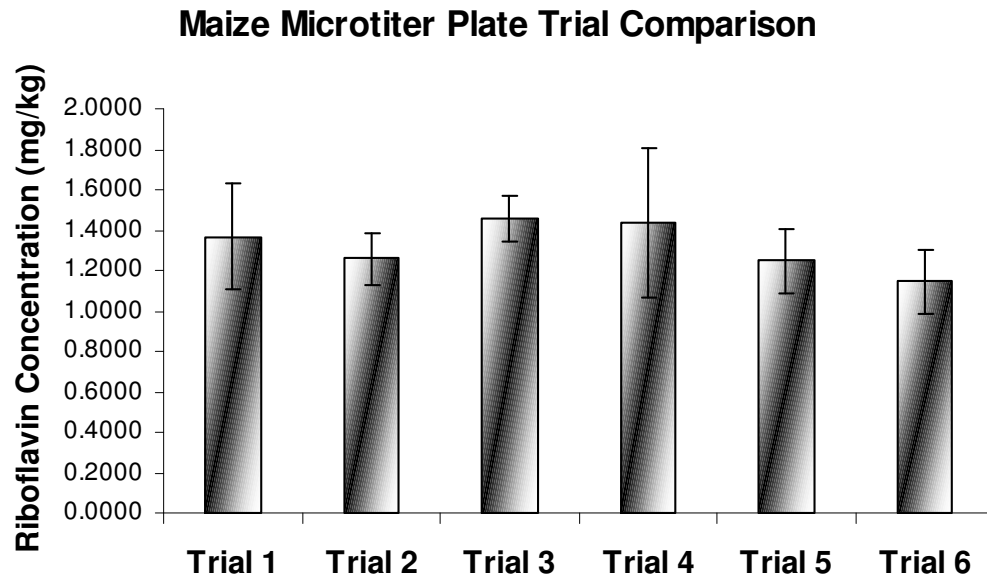


Figure 3.4: Shows the consistency between trials for riboflavin microtiter assay using commercial source of maize for sample using standard deviation. There was no significant difference between trials ($P < 0.05$). Each trial had three replicates.

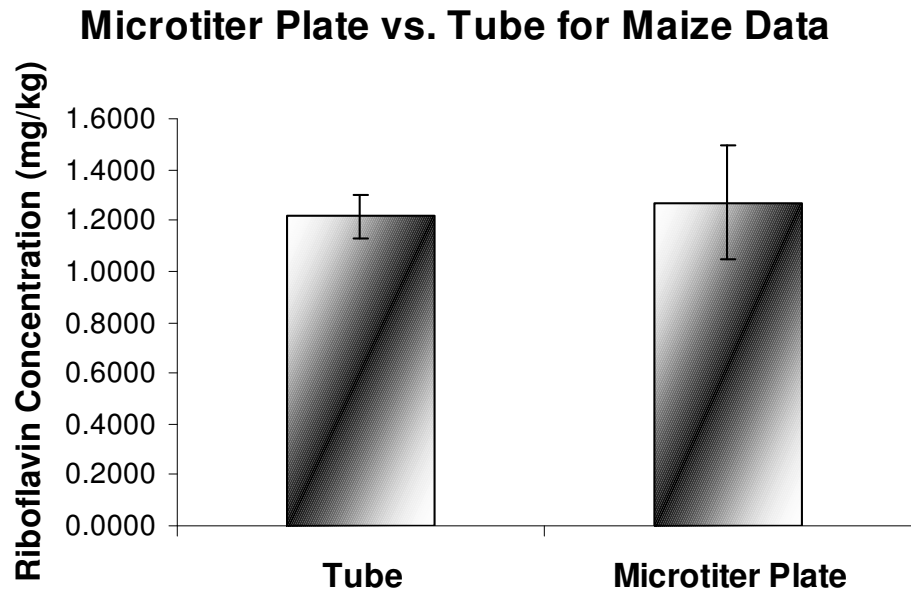


Figure 3.5: Comparison of riboflavin tube assay with the microtiter plate assay using commercial source of maize as sample using standard deviation. There was no significant difference between trials ($P < 0.05$). Six trials were averaged for the values.

precise as the standardized tube assay. To determine if there was a significant difference between the microtiter plate assay and the tube assay, the microtiter plate assay was flavin adenine dinucleotide (FAD) to riboflavin so the *L. rhamnosus* can give an accurate determination of riboflavin (Eitenmiller and Landen, 2000). The final pH of the sample is extremely important because once a certain pH is reached, the riboflavin becomes biologically inactive so an accurate determination of riboflavin the sample is not possible (Ottaway, 2002; Prodanov et al., 2004). The best pH for sample determination that does not reduce the biologically available riboflavin but allows for the optimum growth of *L. rhamnosus* is 6.8 (Ottaway, 2002; Sneath et al., 1986). Once the sample has reached a pH of 6.8, any slight rise in pH will cause the biologically active riboflavin to diminish and become unusable to the bacteria. If bacteria can not utilize the riboflavin, they will not grow, and the sample readings would not be accurate.

The ground soybean meal sample did not show any difference ($P>0.05$) in the of riboflavin quantification between trials. The samples of oatmeal and cornflakes showed some significant differences between samples. This could be due to the fact that the final pH was slightly above the optimum. A comparison between the three trials for ground soybean meal, oatmeal, and cornflakes are depicted in figures 3.6, 3.7, and 3.8 respectively. The samples of rice squares and wheat squares did not show any significant differences of riboflavin between trials (figures 3.9 and 3.10 respectively).

Conclusions

The conversion of the riboflavin assay to a microtiter plate assay was successful. The results showed no statistical difference between the riboflavin quantification when the assay was performed in the tubes versus the microtiter plate. The reproducibility of

the microtiter assay was also successful for maize, cornflakes, rice squares, and wheat squares. There was some differences in the oatmeal and soybean meal trials, but this could be due to slight variations in final pH which can affect riboflavin concentrations.

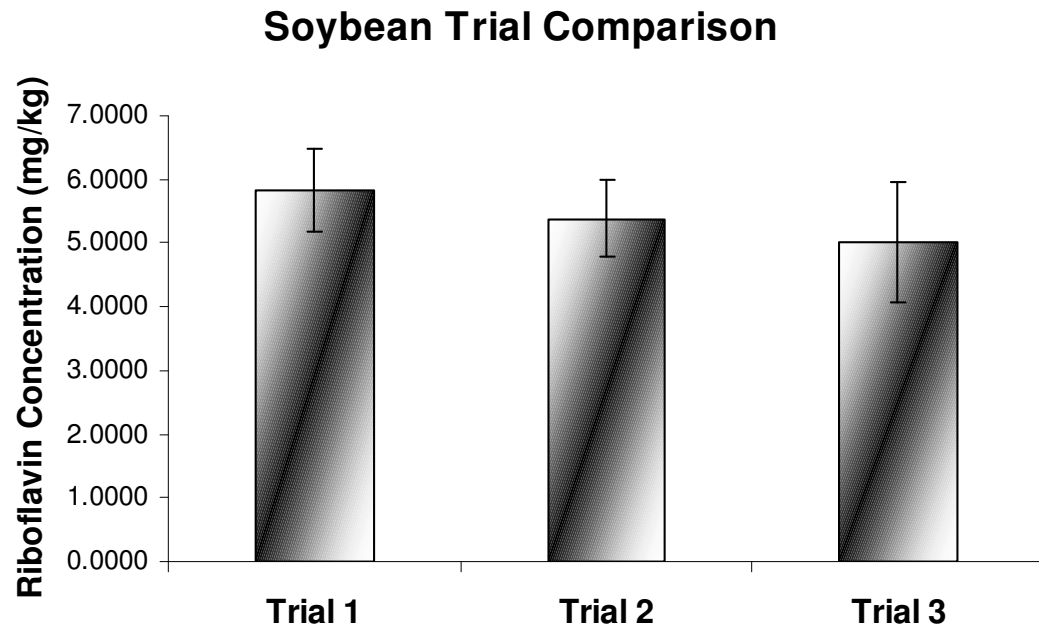


Figure 3.6: Comparison of microtiter plate trials determining riboflavin content in soybeans using standard deviation. There was no significant difference between trials ($P < 0.05$). Each trial had eight replicates.

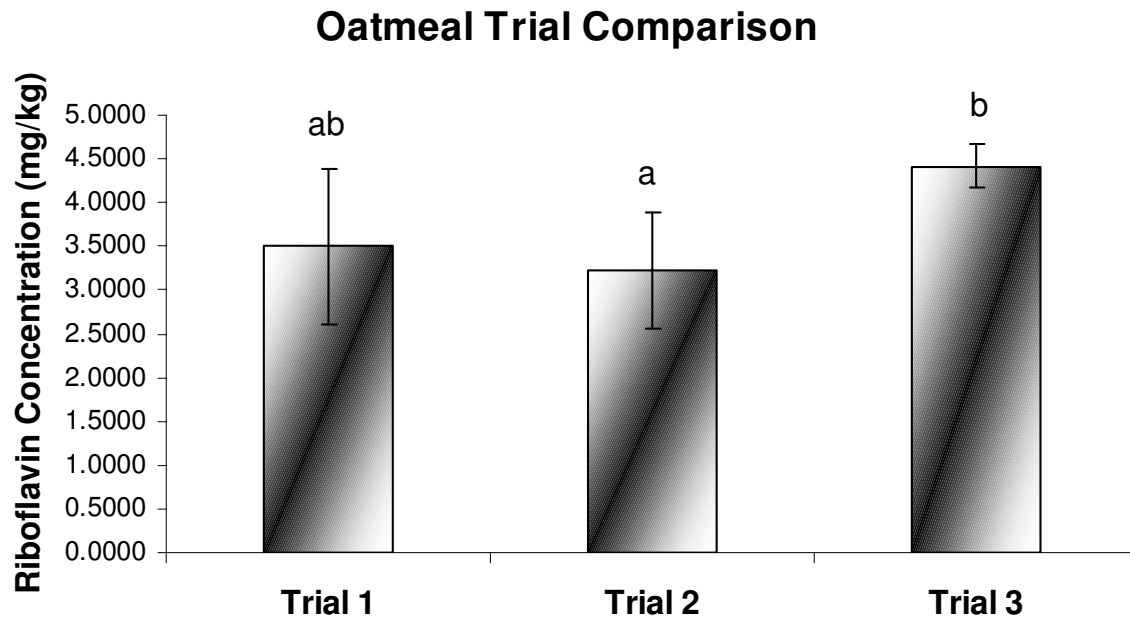


Figure 3.7: Comparison of microtiter plate trials determining riboflavin content in oatmeal using standard deviation. The difference in letters shows statistical difference. Trial 1 is not statistically different from both Trial 2 and 3, but Trials 2 and 3 are statistically different from each other. Each trial had eight replicates

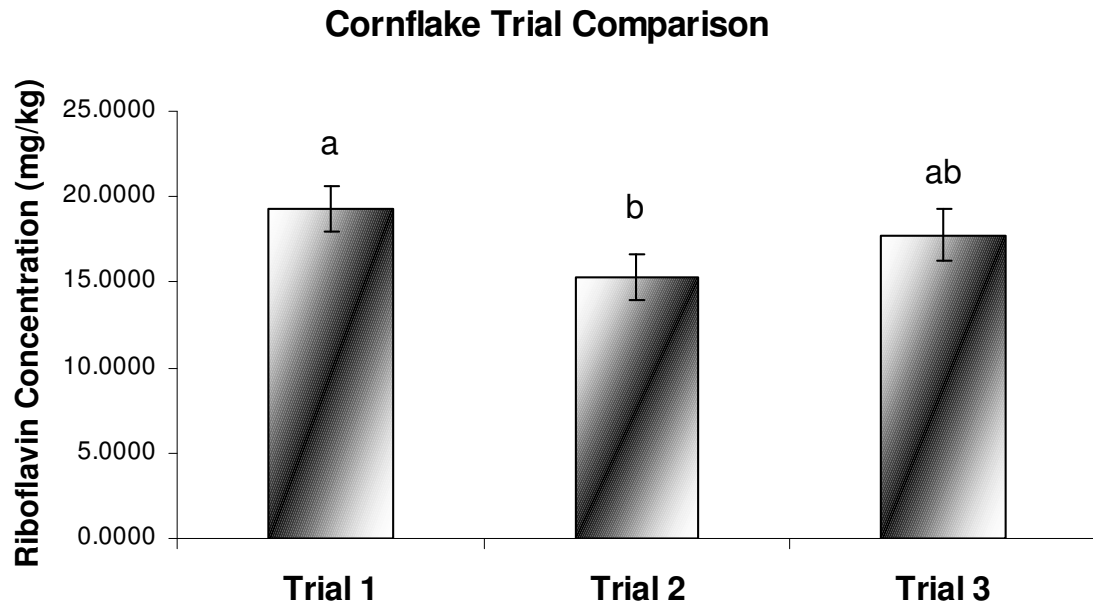


Figure 3.8: Comparison of microtiter plate trials determining riboflavin content in cornflakes using standard deviation. The difference in letters shows statistical difference. Trial 3 is not statistically different from both Trial 1 and 2, but Trials 1 and 2 are statistically different from each other. Each trial had eight replicates.

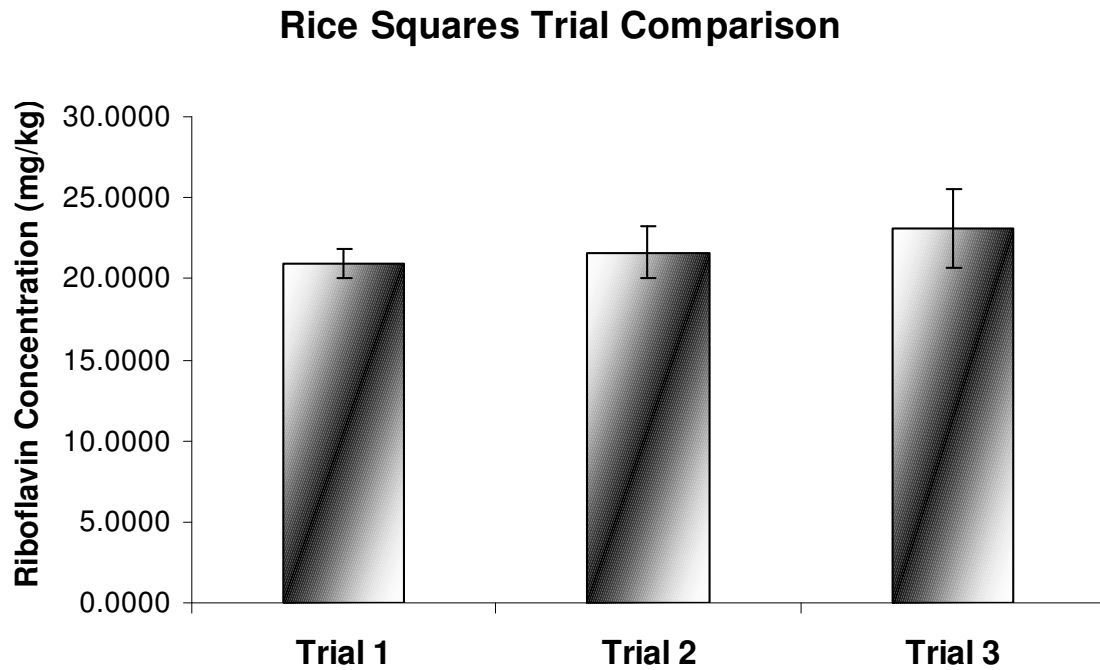


Figure 3.9: Comparison of microtiter plate trials determining riboflavin content in rice squares cereal using standard deviation. There was no significant difference between trials ($P < 0.05$). Each trial had eight replicates



Figure 3.10: Comparison of microtiter plate trails determining riboflavin content in wheat squares cereal using standard deviation. There was no significant difference between trials ($P < 0.05$). Each trial had eight replicates.

CHAPTER IV

ANAEROBIC RIBOFLAVIN ASSAY ADAPTATION

Synopsis

An anaerobic riboflavin microtiter assay was developed to reduce the amount of time for *Lactobacillus rhamnosus* to grow prior to an optical density reading. Anaerobic conditions were incorporated into the assay through the use of an anaerobic hood, and the incorporation the reducing agents ascorbic acid, dithiothreitol, and thioglycolate. The reducing agents were used in various concentrations: 0.05%, 0.075%, and 0.1% (w/v) of ascorbic acid, 0.01%, 0.025%, and 0.05% (w/v) for dithiothritol, and 0.01%, 0.1%, and 0.2% (w/v) for thioglycolate. It was found that the use of an anaerobic hood significantly increased the growth of *L. rhamnosus* during the assay, but it did not reduce the assay time. None of the concentrations of any of the reducing agents had a significant effect on the assay growth.

Introduction

Through food processing, a significant amount of riboflavin can be lost due to heat, moisture, oxygen, pH, and light (Ottaway, 2002). Many cereals are now being fortified with different vitamins including riboflavin which provides a more balanced diet for those people who do not receive enough vitamins through a regular diet (McNulty et al., 1996). To ensure the labels for the fortified foods as well as non-fortified foods have the correct riboflavin content, a rapid riboflavin assay is required. The first step toward accelerating the assay was to convert it from a tube assay to a

microtiter plate assay. The second step is to decrease the amount of time it takes for *L. rhamnosus* to reach the growth point where optical density readings can be taken.

The riboflavin microtiter assay is prepared using a commercial riboflavin assay medium (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). This assay used AOAC assay as a guideline for transferring the riboflavin assay from a tube assay to a microtiter plate assay. The transfer to microtiter plates reduces the time between inoculation of the plate and measurement of absorbance. This bacterium has a significant lag phase during growth when the standardized riboflavin assay is performed. The lag phase requires time for microbial growth and may be due to the aerobic conditions present during initiation of the assay. To reduce the assay, studies using anaerobic conditions were conducted.

L. rhamnosus is a facultative anaerobe which indicates that it is capable of growth in the presence of oxygen or with limited amount of oxygen (Sneath et al., 1986). As a lactic acid bacteria, they are gram-positive, fastidious in their nutritional requirements, have enhanced growth under microaerophilic conditions and produce considerable amounts of lactic acid (Reuter, 1985; Brown, 1968; Tharmaraj and Shah, 2003). For optimum growth, a reduced oxygen content and approximately 10% of CO₂ is recommended (Reuter, 1985).

To reduce assay time even further by reducing the lag phase and accelerating the growth of *L. rhamnosus*, the overall purpose of this study was to determine the effect of anaerobic conditions and reducing agents, at various concentrations in the media, on the riboflavin microtiter plate assay. The anaerobic conditions were achieved by using an anaerobic hood, and the reducing agents studied were ascorbic acid, dithiothreitol, and

thioglycolate. Because *L. rhamnosus* is not a strict anaerobe, the concentration of the reducing agents did not need to create a complete anaerobic environment.

Materials and Methods

Bacterial strain

L. rhamnosus American Type Culture Collection (ATCC) #7469 is the organism used in the riboflavin assays. Stock cultures of *L. rhamnosus* were transferred monthly into stab cultures consisting of 10 mL of Micro Assay Culture Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). They were incubated at 37°C for 24-48 hours and transferred to the refrigerator at 4°C for storage. A working culture was kept on a streak plate and was transferred weekly. The streak plate was stored at 4°C.

To prepare the inoculum for the riboflavin assay, a subculture of the *L. rhamnosus* stock culture was made by inoculating 10 mL of Micro Inoculum Broth (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). The broth was incubated for 16-24 hours at 37°C. The culture was centrifuged using aseptic technique, and the liquid was decanted. The cells were washed three times with 10 mL of 0.85% sterile saline solution and were resuspended in 10 mL of 0.85% sterile saline solution. Using a spectrophotometer at 600 nm, the absorbance of the cell suspension was adjusted to 35-40% transmittance for tubes and 10-15% transmittance for microtiter plates using sterile saline solution to dilute the cell suspension. This cell suspension, 20 µL for tubes and 4 µL for microtiter plates, was used to inoculate broth for the assay. Both the tubes and the micortiter plates were kept at 37°C.

Media preparations

The media was prepared by suspending 4.8 grams of Bacto[®] Riboflavin Assay Medium (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) in 100 mL of deionized water. The medium was boiled 2-3 minutes to dissolve completely. It was then dispensed in 5 mL amounts into tubes. The standard or test sample was added, and the volume was adjusted to 10 mL using deionized water. The tubes were autoclaved for 10 minutes at 121 °C. It was important to keep the riboflavin assay medium at 2-8 °C and to keep the container tightly closed because the medium is very hygroscopic.

Standard curve preparation

The riboflavin stock solution was prepared by weighing 50 mg of riboflavin standard (Sigma) and dissolving in 500 mL of deionized water in a volumetric flask. It was further diluted by adding 0.5 mL to 500 mL of deionized water in a volumetric flask. The diluted stock solution was distributed in the tubes using 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0 mL per tube. These volumes were adjusted to 10 mL with 5 mL of media and deionized water which gave riboflavin concentrations of 0.0, 2.5, 5.0, 7.5, 10, 15, 20, and 30 ng/mL. The tubes were prepared in duplicate. The stock solution for the standard curve was prepared fresh daily to prevent deterioration of riboflavin in the solution.

Culture growth conditions

Cell growth was measured turbidimetrically at 600 nm (A_{600}) on a Milton Roy Spectronic 20D spectrophotometer for the tubes and a Tecan Spectra Flour Plus (Research Triangle Park, NC) for the microtiter plates. The tube assays were allowed to

grow for 18-24 hours as recommended by AOAC while the microtiter plates were allowed to incubate for 14-22 hours. The cells exhibited a lag phase growth period which is defined by the time period between the initiation of growth on the growth curve and exponential phase of the growth curve.

Anaerobic conditions

The anaerobic conditions were achieved through an anaerobic hood as well as the use of reducing agents. A standard curve of each trial was prepared for comparison. One of the microtiter plates was prepared as previously described and placed in the anaerobic hood. It was subjected to the same temperature and time frame for growth as the aerobic standard curve. The microtiter plate from the anaerobic hood was read after six hours for the first absorbance reading. The wells were mixed using a micro pipette to gently pipet the contents up and down of wells to reduce the amount of air introduced into the sample through mixing. After the absorbance reading was taken, the plate was returned to the anaerobic chamber. The first absorbance reading was followed by subsequent readings at two hour intervals which were subject to the same treatment.

The reducing agents incorporated into the media were ascorbic acid, dithiothreitol, and thioglycolate at varying concentrations. The ascorbic acid concentrations were 0.05%, 0.075%, and 0.10%. The dithiothreitol concentrations were 0.01%, 0.025%, and 0.05%. The thioglycolate concentrations were 0.01%, 0.1%, and 0.2%. These percentages were determined using methods described by Levett (1991). A standard curve was prepared as previously described, and the various reducing agents were added to the assay tubes at the desired concentration before they were transferred to

the microtiter plate. The sample microtiter plates were subject to the same temperature and growth conditions as the prepared standard curve.

Statistical analysis

The data generated using the microbiological riboflavin assays were analyzed using regression analysis. All statistical analysis were considered significant at $P < 0.05$. The statistical data was generated using Statistical Analysis Software 8.0 (SAS) (SAS Institute, Cary, NC, USA).

Results and Discussion

Growth responses of *L. rhamnosus* to anaerobic conditions at various times are depicted in figures 4.1 through 4.4. The aerobic versus anaerobic trials were performed three times, and the results were averaged for the respective standard riboflavin concentrations. To determine if there was a significant difference between the two growing conditions, each standard curve was graphed at the varying times of optical density readings and regression analysis was performed. The times that optical density readings were taken were 8, 10, 12, and 14 hours after inoculation. The readings were not performed more this because the microtiter plates had to be exposed to oxygen every time the plate was taken from the anaerobic hood. Also, to reduce the amount of air incorporated into the inoculated media, the cell suspension was mixed using pipets.

There was a significant difference in the growth response for absorbance readings taken at hours 8 through 14 compared to the standard that was not subjected to anaerobic conditions. The results from the linear regression analysis confirmed significant differences in the OD values between the aerobic and anaerobic conditions. Although

Riboflavin Microtiter Plate Assay at 8 Hours

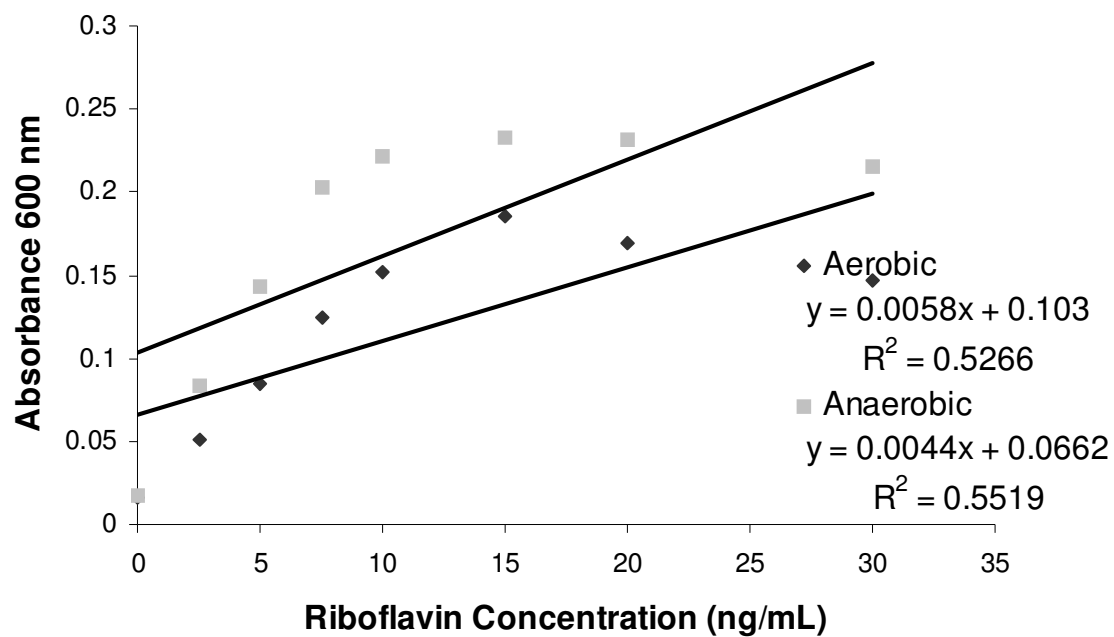


Figure 4.1: Comparison of aerobic growth and anaerobic growth of the riboflavin assay standard curve after 8 hours. Each concentration is an average of eight replicates.

Riboflavin Microtiter Assay at 10 Hours

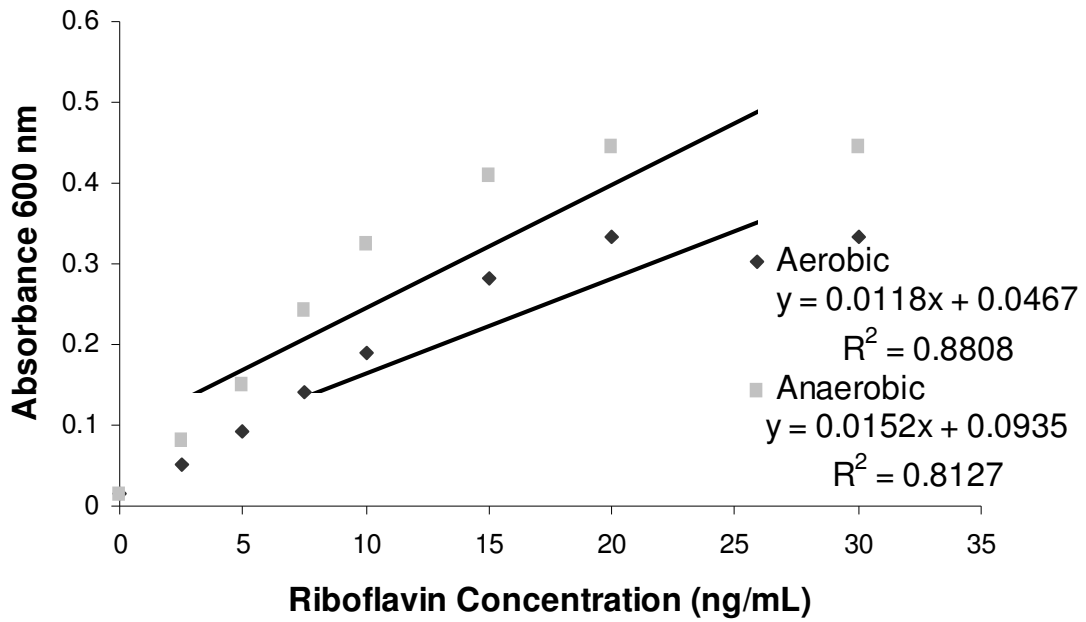


Figure 4.2: Comparison of aerobic growth and anaerobic growth of the riboflavin assay standard curve after 10 hours. Each concentration is an average of eight replicates.

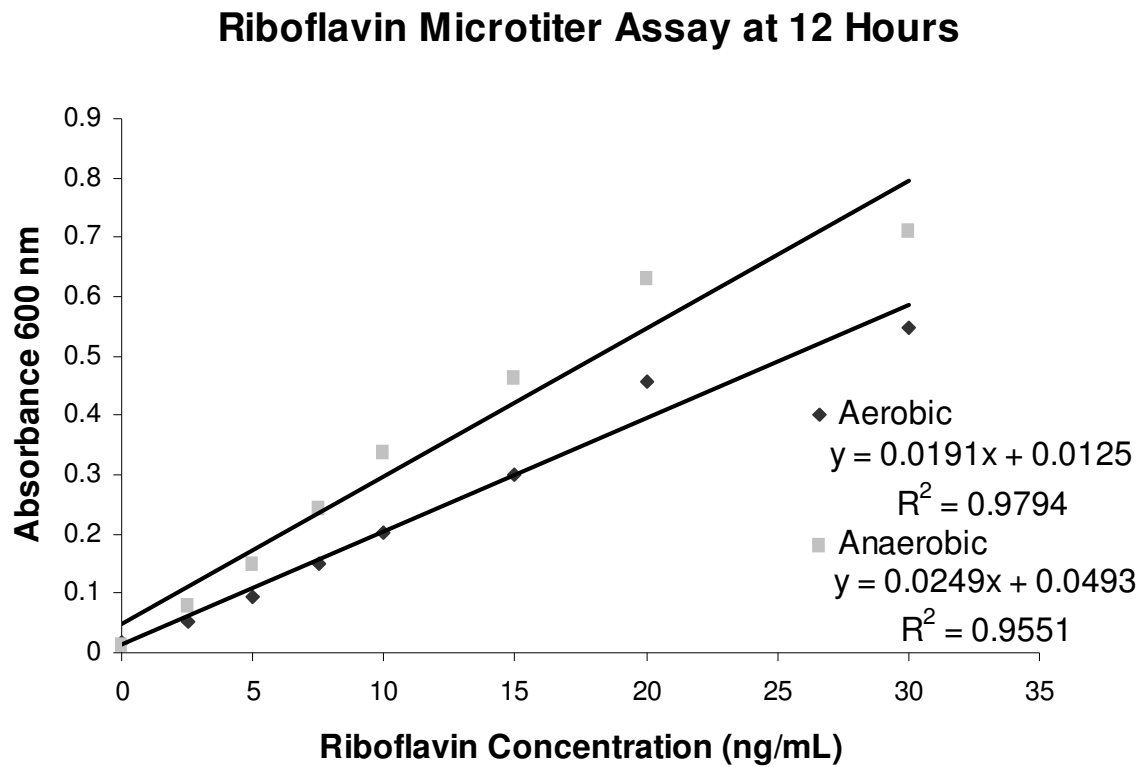


Figure 4.3: Comparison of aerobic growth and anaerobic growth of the riboflavin assay standard curve after 12 hours. Each concentration is an average of eight replicates.

Riboflavin Microtiter Plate Assay at 14 Hours

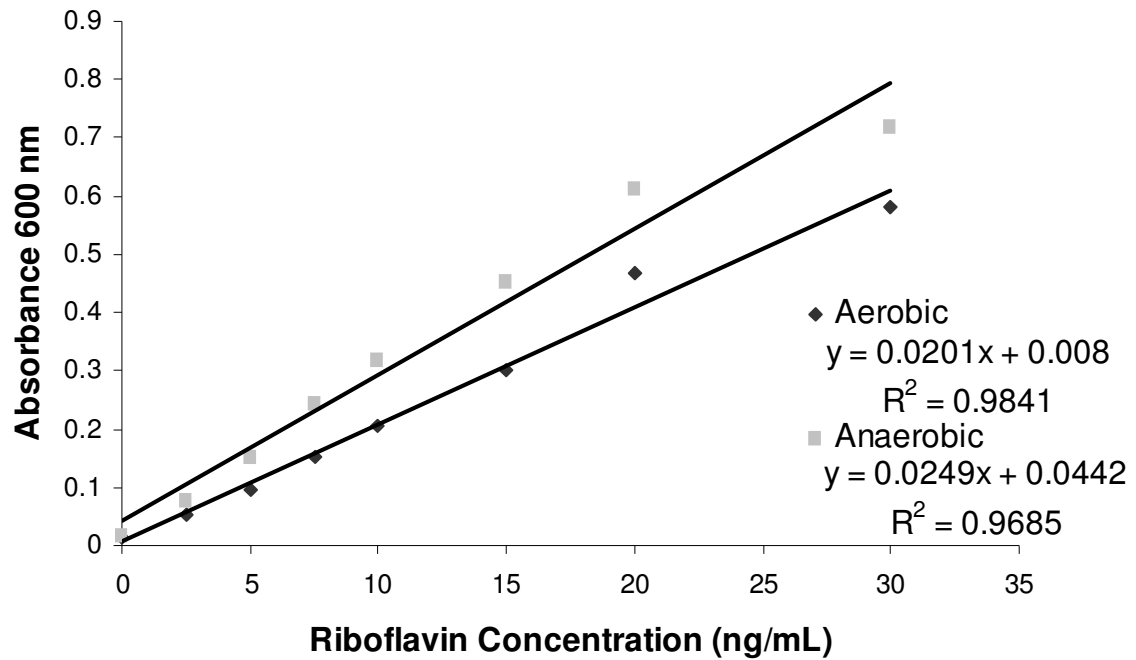


Figure 4.4: Comparison of aerobic growth and anaerobic growth of the riboflavin assay standard curve after 14 hours. Each concentration is an average of eight replicates.

the cells for each riboflavin concentration were greater in the anaerobic conditions, the rate of growth was the same. The growth curve for the anaerobic standard compared to the aerobic standard is presented in figure 4.5 which showed that the slopes of the anaerobic and aerobic regression lines are the same. The purpose of this study was to determine if results could be obtained more rapidly by decreasing the lag phase of the bacteria. This was not the case since the growth rates of bacteria subjected to anaerobic and aerobic conditions are the same. The study does illustrate that the total growth measured by the OD value does increase with anaerobic conditions, but the growth rate does not change. The assay reached maximum growth after 14 hours post inoculation whether subjected both anaerobic or aerobic conditions.

Ascorbic acid, also known as vitamin C, is a natural antioxidant and protects against free radical damage (Hathcock et al., 2005). This compound was chosen for use in this study because it is non-toxic to humans and is a proven reducing agent (Smith and Pierson, 1979). Dithiothreitol and thioglycolate, although they have minor warnings about skin and eye irritation, are relatively safe to work with when gloves are used (Nebra et al., 2002). They are also proven reducing agents, and therefore, chosen for incorporation into the riboflavin assay media (Nebra et al., 2002; Kirakosyan et al., 2004).

Studies by Smith and Pierson (1979) proved that ascorbic acid and thioglycolate were able to reduce the amount of oxygen in media while Kirakosyan et al.(2004) demonstrated the ability of dithiothreitol to also reduce the amount of oxygen present in the prepared media. Three trials were conducted for each concentration of each reducing agent. The three trials for each reducing agent were averaged and the average natural log of the absorbance graphed over time to generate a growth curve for each concentration.

The standard curves that were generated from varying concentrations of reducing agents did not show a significant difference in growth response (figures 4.6 through 4.8). These graphs, as well as the linear regression analysis, show that the growth of *L. rhamnosus* when inoculated into media containing the various reducing agents was not affected in neither a positive nor negative manner when compared to the unmodified riboflavin standard curve.

Conclusions

Although the anaerobic adaptations increased the amount of growth, the growth rate did not increase as expected. The anaerobic adaptation was not effective at accelerating growth whether the *L. rhamnosus* was grown under aerobic or anaerobic conditions in microtiter plates. The reducing agents did not prove to affect the growth of *L. rhamnosus* in any manner. The addition of reducing agents in the media had no effect on growth rate.

Growth Curve of Anaerobic vs. Aerobic

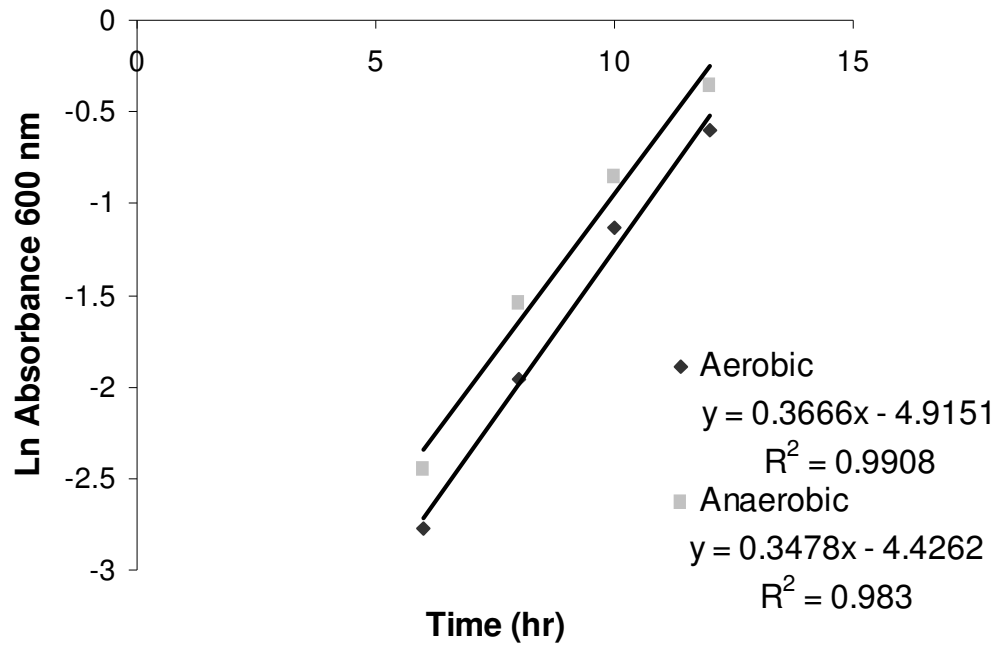


Figure 4.5: Comparison of aerobic growth and anaerobic growth of the riboflavin assay standard curve using the natural log of the average of optical density readings at a riboflavin concentration of 30 ng/mL. The graph shows no significant difference between the slopes of the two trials ($P < 0.05$). Each concentration is an average of eight replicates.

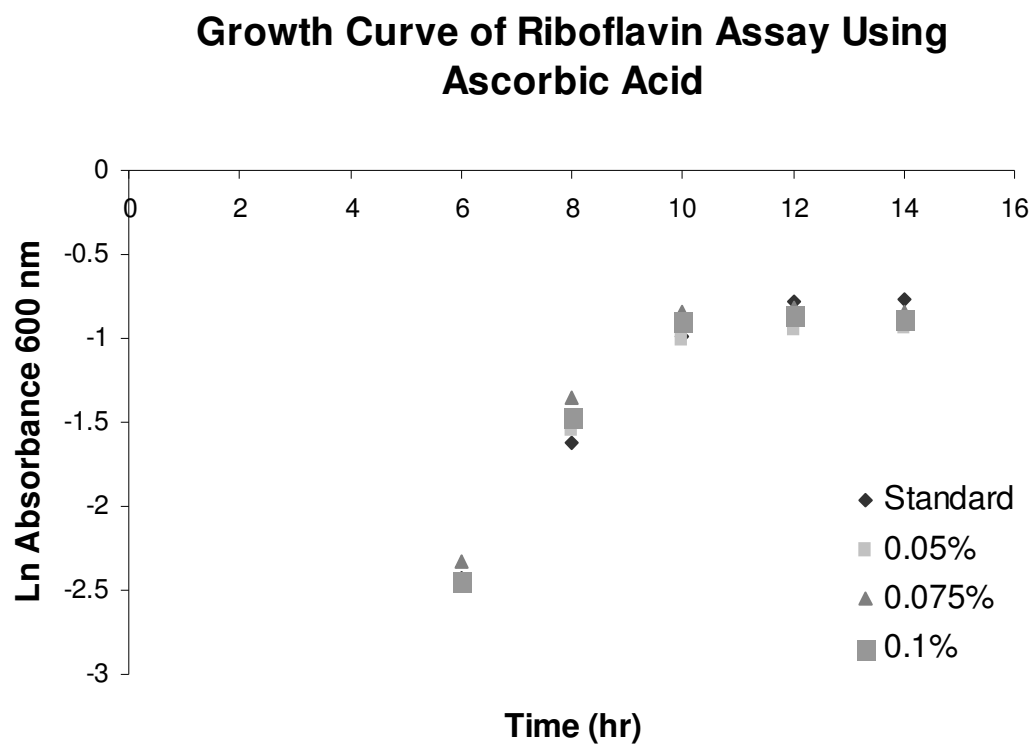


Figure 4.6: Comparison of growth curves using concentrations of ascorbic acid ranging from 0.05% to 0.1% against the riboflavin standard curve. Each concentration is an average of eight replicates.

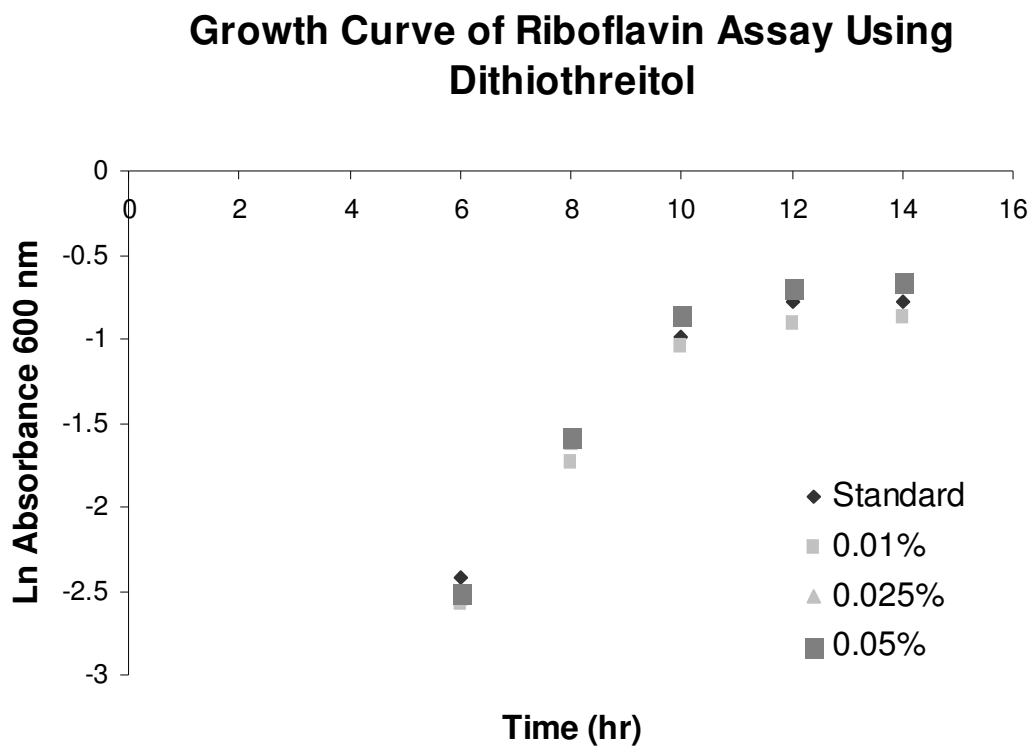


Figure 4.7: Comparison of growth curves using concentrations of dithiothreitol ranging from 0.01% to 0.05% against the riboflavin standard curve. Each concentration is an average of eight replicates.

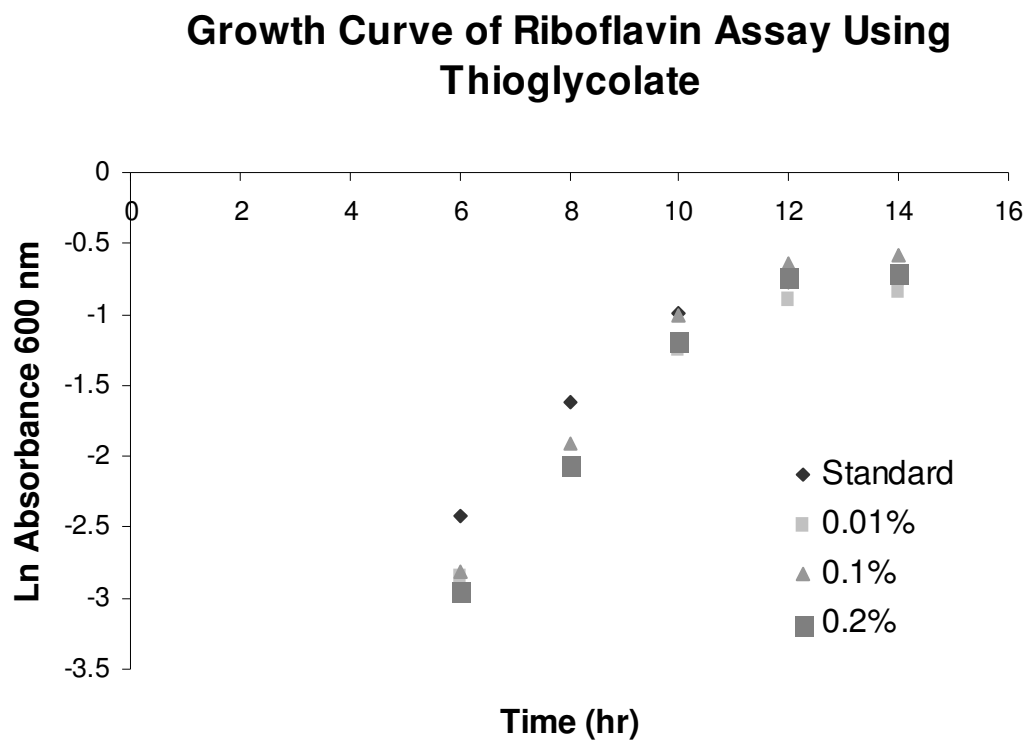


Figure 4.8: Comparison of growth curves using concentrations of thioglycolate ranging from 0.01% to 0.2% against the riboflavin standard curve. Each concentration is an average of eight replicates.

CHAPTER V

CONCLUSIONS

The development of a more rapid microtiter riboflavin assay using *L. rhamnosus* was presented. This assay is important for the food industry to allow for more rapid determination of riboflavin in food sources and enable more samples to be analyzed at one time. Riboflavin is an essential part of the diet and is incorporated into many metabolic functions of the body. Deficiencies can be a problem in regions of the world where the main staple is rice. To prevent and cure riboflavin deficiencies, many cereal grains are being fortified with riboflavin. With the fortification of riboflavin in many cereal grains and the detrimental effects of certain processing procedures on riboflavin concentration, verifying riboflavin content in these foods is especially important.

The objective of this study was to modify the accepted AOAC riboflavin tube assay by scaling down to microtiter plates and evaluating the anaerobic conditions to reduce the lag phase of *L. rhamnosus*. The goal of these changes was to produce a more rapid assay and save on test materials.

The microtiter plate adaptation required four hours less time as compared to the tube assay. The final incubation time from the time of inoculation until the final reading was reduced from 18 hours down to 14 hours. The precision of the assay was determined using a commercial maize sample. A comparison between the AOAC approved tube assay and the microtiter plate assay showed no significant differences between the methods. Other samples were also assayed for riboflavin concentration to determine the reproducibility of the microtiter plate riboflavin assay. Commercial soybean meal, rice squares, and wheat squares all showed consistency between trials

with no significant differences in riboflavin concentration. Cornflakes and oatmeal showed some differences between trials. This could be caused by the pH being slightly higher than the optimum 6.8. Riboflavin begins to become biologically inactive above a pH of 6.8. The higher the pH rises, the more biologically inactive it becomes.

Samples incubated under anaerobic conditions showed an increase in cell number, but the growth rate was not significantly affected. Because growth rate was not affected by the anaerobic conditions, the final assay time were equivalent. Reducing agents, ascorbic acid, dithiothreitol, and thioglycolate, added to the media did not affect the growth of *L. rhamnosus* in any fashion. Growth remained constant between the control and the treated samples containing the various concentrations of reducing agents. Neither the anaerobic conditions nor the reducing agents reduced assay time for the microtiter plate assay.

Use of the microtiter plate assay for the determination of riboflavin in food samples, reduces the assay time by about four hours. This improvement saves not only time and materials, but enables a larger number of assays to be performed.

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Symposia Presentations

- I. **J. L. Golbach**, C. L. Woodward, V. I. Chalova, and S. C. Ricke. 2004
Decreasing the Time Required for the Auxotroph *Lactobacillus*
rhamnosus Assay by Adaptation to Microtiter Plates. Poultry Science
Association Annual Meeting, St. Louis, Missouri.
- II. **R.S. Hardin**, M.M. Kundiger, C.L. Woodward, L.M. Donalson, J.L.
Golbach, and S.C. Ricke. 2004. Development of Quality Control
Laboratory Design Project for Poultry Science Undergraduate Students
Enrolled in Advanced Food Microbiology Course. Poultry Science 83:76.

Professional Appointments

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